PCT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 01 November 1999 (01.11.99)	
International application No. PCT/GB99/00929	**Applicant's or agent's file reference** PBA/DO88126PWO
International filing date (day/month/year) 24 March 1999 (24.03.99)	**Priority date** (day/month/year) 25 March 1998 (25.03.98)
Applicant MULROONEY, Conor et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 September 1999 (02.09.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer C. Carrié
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

REC'D 27 JUN 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PBA/DO88126PWO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/00929	24/03/1999	25/03/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant TEPNEL MEDICAL LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/09/1999	21.06.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Renggli, J Telephone No. +49 89 2399 7461

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/00929

I. Basis of th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-21 as originally filed

Claims, No.:

1-20 as originally filed

Drawings, sheets:

1/13-13/13 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-20
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-20
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

ITEM I:

4. Additional observations:

Sequence listing filed on 17.08.99, received on 20.08.99, pages 1/3-3/3: Under Rule 13ter.(f) PCT, sequence listings filed after the filing date of the application do not form part of the description and will not be annexed to this report.

ITEM V:

1. Reference is made to the following documents:

D1 EP-A-0 500 224
D2 WO 97/04126

2. Industrial applicability (Art. 33(4) PCT):

The subject-matter of claims 1-20 is susceptible of industrial application.

3. Novelty (Art. 33(2) PCT):

D1 concerns an isothermal and sensitive method for e.g. detecting a target nucleic acid in a sample (cf D1, c3, l. 50-53; c5, l. 21-33). The method of D1 is based on the use of (a) a strand displacing nucleic acid polymerase, (b) dNTPs, at least one being exonuclease resistant, (c) a double strand specific 5'-3' exonuclease and (d) at least one unmodified primer specific for the 3' region of the single stranded target fragment (cf. D1, c. 5, l. 36-c. 6, l. 2; c. 7, l. 4-8; c. 7, l. 18-25; c. 8, l. 15- c. 9, l. 17; figs. 1 and 2). After amplification, the amplified target sequence (see fig. 2) containing exonuclease resistant nucleotides can be detected by any known method in the field (cf. D1, c. 6, l. 3-40; c. 9, l. 49-58); alternatively, probes can be generated (cf. D1, c. 6, l. 41-51; c. 10, l. 1-13).
Claims 1, 19 and 20 from the present application differs from D1 in that (i) 1st and 2nd primers containing a 5' double strand exonuclease resistant portion are used in combination with (ii) 3rd and 4th primers partially homologous to the digestible portion of the 1st and 2nd primers. Claims 1-20 of the present application are

therefore novel within the meaning of Article 33(2) PCT.

D2 is directed to a sensitive and isothermal method for amplifying/detecting a target nucleic acid (cf. D2, pages 2-5). The method of D2 is based on the use of strand displacement amplification in combination with chimeric primers comprising a RNA portion (cf. D2, page 7 and page 9, line 27-page 11, line 15; figs 1-6 and corresponding parts of the description). The method of D2 requires the presence of the following enzymatic activities: (i) SDA, (ii) RNAse H and (iii) DNA polymerase (cf. D2, page 14, line 21-page 16, line 10). The applications of D2 are similar to the ones of D1 (cf. D2, pages 22-24). None of the 1st and 2nd primers disclosed in D2 contains a 5' double strand exonuclease resistant portion and the said 1st and 2nd primers are consequently not used in combination with 3rd and 4th primers homologous to the digested portion. Claims 1-20 of the present application are therefore novel over D2 (Art. 33(2) PCT).

4. Inventive step (Art. 33(3) PCT):

D2 is considered as the closest prior art document. It discloses a multi-primer's pairs method enabling the amplification of target sequences (see D2, figures). The first and second primers of D2 are chimeric DNA/RNA molecules. The said primers are associated with certain disadvantages (see description of the present application, page 3, lines 3-17).

The problem solved by claims 1, 19 and 20 can therefore be seen as the provision of a more convenient method for the amplification of a target sequence (see description of the present application, figs. 3 and 4).

The common solution consists in the use of 1st and 2nd primers containing a 5' exonuclease resistant portion in the presence of an enzyme having 5' double stranded specific exonuclease activity, in combination with 3rd and 4th primers homologous to the digested portion of 1st and 2nd primers (see description of the present application, figs. 3 and 4).

None of the prior art document cited in the ISR contains any suggestion leading to this solution. D1 contemplates the use of exonuclease resistant nucleotides for

producing an exonuclease resistant target sequence, but does not suggest the use of primers containing the said resistant portion in combination with 3rd and 4th primers enabling the exponential amplification of the target sequence (see description of the present application, pages 2-3, bridging paragraph).

Claims 1-20 of the present application are therefore regarded as inventive within the meaning of Article 33(3) PCT.

ITEM VIII:

1. The subject-matter of claim 2 is unclear (Art. 6 PCT). The steps necessary for conducting the method of claim 2 "in situ" are not clearly identified (see the PCT Guidelines, PCT Gazette-Section IV, III-4.1 and 4.4). Moreover, it is not clear what the term "in situ" encompasses and especially, it should have been made clear that methods carried out in vivo are excluded from the scope of this claim (Rule 67.1, iv) PCT).

2. Although claims 1 and 19 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought. The aforementioned claims therefore lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.

   Hence, claims 1 and 19 do not meet the requirements of Article 6 PCT. In addition, claim 20 differs from the said claims only in that exonuclease resistant nucleotides are used. Consequently, this latter claim could have been made dependent (Rule 6.4 PCT).

3. The wording "at least a portion" used in claims 7 and 8 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

4. The wording "a partial degree of resistance to digestion" used in claim 15 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

5. The use of brackets in the claims should have been avoided, see claim 16, Art. 6 PCT.

6. The features of claims 6 and 16 are not referred to in the description. Claims 6 and 16 are therefore not supported by the description as required by Article 6 PCT.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PBA/D088126PWO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 00929	24/03/1999	25/03/1998
Applicant		
TEPNEL MEDICAL LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1, 2, 3

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]GB 99/00929

A. CLASSIFICATION OF SUBJECT MATTE[redacted]
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 500 224 A (BECTON DICKINSON CO) 26 August 1992 (1992-08-26) cited in the application see whole doc. esp., col.7 l.4 ff. and figures ---	1-20
Y	WO 97 04126 A (BIO MERIEUX ;CLEUZIAT PHILIPPE (FR); MANDRAND BERNARD (FR)) 6 February 1997 (1997-02-06) cited in the application see whole doc. esp, claims and figs. ---	1-20
Y	WO 94 16090 A (KNAPP MICHAEL R ;NIKIFOROV THEO (US); MOLECULAR TOOL INC (US)) 21 July 1994 (1994-07-21) see whole doc. esp. claims --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1999	27/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 497 272 A (BECTON DICKINSON CO) 5 August 1992 (1992-08-05) the whole document	
A	WALKER G T ET AL: "STRAND DISPLACEMENT AMPLIFICATION - AN ISOTHERMAL, IN VITRO DNA AMPLIFICATION TECHNIQUE" NUCLEIC ACIDS RESEARCH, vol. 20, no. 7, 1 January 1992 (1992-01-01), pages 1691-1696, XP002019521 ISSN: 0305-1048	

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
P[redacted]B 99/00929

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0500224 A	26-08-1992	AU 8997991 A	06-08-1992
		CA 2058567 A	01-08-1992
		JP 5130870 A	28-05-1993
WO 9704126 A	06-02-1997	FR 2737223 A	31-01-1997
		CA 2200627 A	06-02-1997
		EP 0787209 A	06-08-1997
		US 5824517 A	20-10-1998
WO 9416090 A	21-07-1994	US 5518900 A	21-05-1996
		AU 674211 B	12-12-1996
		AU 6126294 A	15-08-1994
		CA 2153898 A	21-07-1994
		EP 0679190 A	02-11-1995
		JP 8505535 T	18-06-1996
		US 5762876 A	09-06-1998
EP 0497272 A	05-08-1992	US 5455166 A	03-10-1995
		AT 128737 T	15-10-1995
		AU 652214 B	18-08-1994
		AU 1026492 A	06-08-1992
		AU 659429 B	18-05-1995
		CA 2060371 A	01-08-1992
		CA 2060372 A	01-08-1992
		DE 69205181 D	09-11-1995
		DE 69205181 T	21-03-1996
		DK 497272 T	29-01-1996
		ES 2077887 T	01-12-1995
		GR 3018271 T	29-02-1996
		JP 2076096 C	25-07-1996
		JP 5192195 A	03-08-1993
		JP 7114718 B	13-12-1995
		US 5712124 A	27-01-1995
		AU 1069992 A	29-07-1993
		BR 9300028 A	28-09-1993
		KR 9503619 B	17-04-1995
		MX 9300069 A	01-07-1993

PCT

LD INTELLECTUAL PROPERTY ORGANIZATIC
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A2

(11) International Publication Number: WO 99/49081

(43) International Publication Date: 30 September 1999 (30.09.99)

(21) International Application Number: PCT/GB99/00929

(22) International Filing Date: 24 March 1999 (24.03.99)

(30) Priority Data:
9806253.2 25 March 1998 (25.03.98) GB

(71) Applicant *(for all designated States except US)*: TEPNEL MEDICAL LIMITED [GB/GB]; Unit 8, St. George's Court, Hanover Business Park, Altrincham WA14 SVA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MULROONEY, Conor [GB/GB]; Flat 8, 25 Ladybridge Road, Cheadle Hulme, Cheshire SK8 5DL (GB); OULTRAM, John, Douglas [GB/GB]; 27 Woodsend Road South, Flixton, Manchester M41 6QB (GB).

(74) Agent: ATKINSON, Peter, Birch; Marks & Clerk, Sussex House, 83–85 Mosley Street, Manchester M2 3LG (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

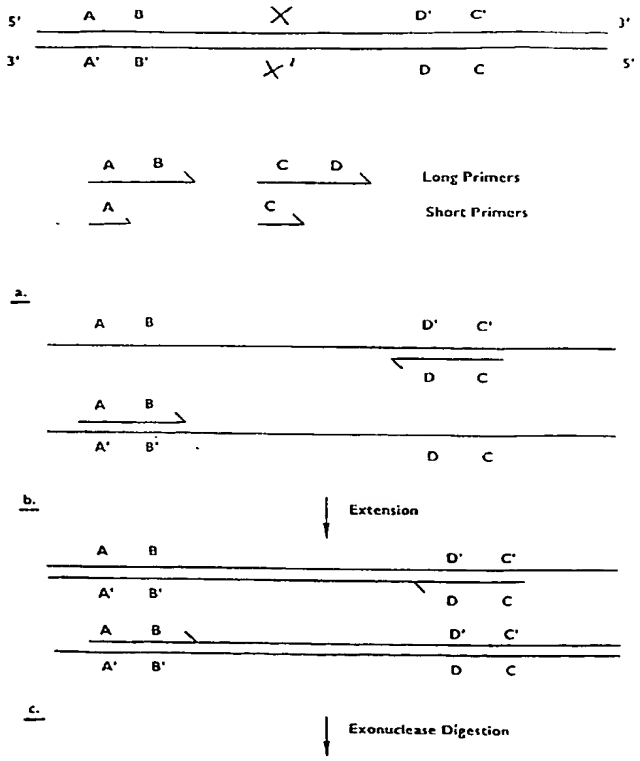
Published

Without international search report and to be republished upon receipt of that report.

(54) Title: AMPLIFICATION OF NUCLEIC ACIDS

(57) Abstract

A method of amplifying complementary first and second nucleic acid sequences each of which has a binding region at its 3' end. The method conmprises treating the separated single stranded sequences with (a) first and second primers each capable of hybridising to the 3'–binding regions of the first and second strands respectively and each including remote from its 5'–end a digestion resistant region which, with the primer hybridized to the complementary 3'–binding region, allows only partial digestion of the primer by the enzyme (d) having 5'–double strand specific exonuclease activity, (b) third and fourth primers each having a degree of sequence homology with the partially digestible regions of the first and second primers respectively whereby the third and fourth primers are capable of hybridising to the 3'–binding regions of the first and second strands respectively, (c) an enzyme having strand displacing polymerase activity, (d) an enzyme having 5' double stranded specific exonuclease activity, said enzyme (d) possibly being provided by enzyme (c) in the case where the latter also has the required exonuclease activity, and (e) nucleoside triphosphates. The reaction is conducted under conditions permitting hybridisation, exonuclease digestion and strand displacement polymerisation thereby producing an amplified amount of the first and second strands.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

AMPLIFICATION OF NUCLEIC ACIDS

The present invention relates to the amplification of nucleic acids, i.e. procedures for producing copies of nucleic acid sequences.

As used herein the term "nucleic acid" includes protein nucleic acid (PNA) (i.e. nucleic acids in which the bases are linked by a polypeptide backbone) as well as the naturally occurring nucleic acids (e.g. DNA and RNA), or analogues thereof, having a sugar phosphate backbone.

Various nucleic acid amplification techniques are already known, e.g. the Polymerase Chain Reaction (PCR). However many of these techniques (including PCR) suffer from the disadvantage that various cycles of heating and cooling are required for each amplification reaction. Thus, in a typical amplification reaction, the sequence (in single stranded form) to be amplified is treated with an oligonucleotide capable of hybridising to the sequence at a particular location thereof, the treatment being effected at a temperature (and under other conditions, e.g. buffers etc.) at which the hybridisation will occur. In the next step (which may or may not be effected at the same temperature) a polymerase enzyme is used to extend the oligonucleotide primers (using the original sequence as a template) to produce a strand which is complementary to the original strand and which is hybridised thereto. Subsequently the reaction mixture must be heated to denature the complementary strands and then cooled so that the above described procedure (i.e. primer hybridisation, extension, denaturing) may be repeated. A particular amplification reaction may require many repeats of the cycle before a sufficient quantity of the nucleic acid is generated (e.g. for the purposes of a diagnostic test or for research purposes). The need to "temperature cycle" the reaction many times is a considerable disadvantage.

Several alternative amplification strategies are known which can be performed at a single temperature. Examples of such isothermal technologies include Strand Displacement Amplification (SDA), (G. T. Walker; US Patent No. 5455166).

SDA takes advantage of the ability of certain restriction enzymes to cleave hemi-modified restriction sites only on the un-modified DNA strand. By using non-modified primers containing such a restriction site, and including modified dNTP analogues, it is possible to introduce single strand nicks into the primer when it is rendered double stranded by DNA which has been synthesised to include modified nucleotides. The introduction of this nick allows a strand displacing polymerase to extend the sequence upstream of the nick by displacing downstream sequences into solution where they are picked up by primers directed to the other end of the strand to be amplified. The process is rapid and can be performed at a single temperature. However, SDA requires specialised primers, which are not wholly specific to the natural target DNA sequence to which they are directed. This arises from the need to introduce into the sequence to be amplified restriction sites at the ends of the molecule for the reaction to occur. Furthermore, a degree of manipulation is required to initiate the amplification reaction. For instance, a second set of primers are required to displace the extended first copies into solution so that the second amplification primer can act upon the single strand, displaced, extension product.

Another isothermal amplification method known in the art is Exonuclease Mediated Strand Displacement Amplification (G. T. Walker, EP-A-0 500 224). This technique takes advantage of the ability of thiol-substituted nucleotide analogues to protect downstream sequences from digestion by a 5' double strand specific exonuclease. In this method unmodified primers are used together with a 5' double strand specific exonuclease, a strand displacing DNA polymerase and an excess of deoxynucleosidetriphosphates, at least one of which is substituted to provide resistance to exonuclease activity. The method as described, however, will not provide for exponential amplification as the loss of unmodified primer sequences (by

exonuclease activity) precludes the copying of those same sequences, to allow hybridisation at the complementary sequence, later in the reaction

In another amplification method (P. Cleuziat, WO-A-97/04126) chimeric RNA\DNA primers are used, together with shorter DNA primers, a strand displacing polymerase, an excess of deoxynucleosidetriphosphates and RNAase H. Binding of the chimeric primer to its target generates a short, double-stranded, DNA/RNA hybrid from which the RNA portion is cleaved by the action of RNAse H. This reveals a site for the shorter DNA primer which hybridises and is extended by the action of the DNA polymerase. A disadvantage of this system is the need for the RNA carrying primers. RNA is notoriously sensitive to degradation by ribonucleases which are abundant contaminants of many environments, and, extensive decontamination procedures must be observed to limit this effect during RNA manipulations. Because of the low turnover rate of the RNase H enzyme, longer reaction times must be used or the enzyme must be present at a higher concentration. Also, the amplification must be performed in the abiding presence of non-target sequences, present in the sample, which are known to contribute to anomalous results during other amplification reactions, such as PCR.

It is an object of the present invention to provide an amplification method which may be effected under isothermal conditions, if required, and can amplify the natural sequence entirely, if required.

According to the present invention there is provided a method of amplifying complementary first and second nucleic acid sequences each of which has a binding region at its 3' end, the method comprising treating the separated single stranded sequences with

(a) first and second primers each capable of hybridising to the 3'-binding regions of the first and second strands respectively and each including remote from its

5'-end a digestion resistant region which, with the primer hybridised to the complementary 3'-binding region, allows only partial digestion of the primer by the enzyme (d) having 5'-double strand specific exonuclease activity,

(b) third and fourth primers each having a degree of sequence homology with the partially digestible regions of the first and second primers respectively whereby the third and fourth primers are capable of hybridising to the 3'-binding regions of the first and second strands respectively,

(c) an enzyme having strand displacing polymerase activity,

(d) an enzyme having 5' double stranded specific exonuclease activity, said enzyme (d) possibly being provided by enzyme (c) in the case where the latter also has the required exonuclease activity, and

(e) nucleoside triphosphates,

under conditions permitting hybridisation, exonuclease digestion and strand displacement polymerisation thereby producing an amplified amount of the first and second strands.

As used herein, the term 5'-double stranded specific exonuclease activity means that the principal exonuclease activity is double stranded activity whilst not precluding the possibility of a degree of single stranded activity. The term 5'-double stranded specific exonuclease is to be interpreted accordingly.

The present invention thus provides a method for amplification of a nucleic acid molecule. The complementary sequences on which the reaction is effected may be generated (e.g. *in situ* in the reaction) from a single stranded molecule by methods well known in the art.

The method may be effected under conditions such that all of reactants (a)-(e) are simultaneously present in a reaction mixture or under conditions in which the reagents are used sequentially as appropriate.

The method utilises a 5' double stranded specific exonuclease, i.e. an exonuclease which will digest the strands of a double stranded molecule from the 5' ends thereof. The method also requires the use of an enzyme having strand displacing polymerase activity. The method of the invention may be performed with two enzymes, one being a 5' double stranded specific exonuclease and the other being a strand displacing polymerase. It is however also possible to use a single enzyme having both of these activities.

The invention further utilises the first and second primers each of which incorporates a region (e.g. provided by modified nucleotides, see below) resistant to digestion by the 5' double stranded specific exonuclease. It should however be understood that the terms "first" and "second" primers are used in an explanatory sense to identify primers which hybridise to the 3' binding regions of the first and second strands respectively. The terms do not necessarily imply that the primers are different since it is possible to envisage embodiments of the invention in which the 3' binding regions of the first and second strands have sufficient sequence identity for the "first" and "second" primers to be one and the same.

The method further utilises the third and fourth primers which need not incorporate modified nucleotides and which have a degree of sequence homology with the partially digestible regions of the first and second primers. In certain embodiments of the invention, the "third" and "fourth" primers may be identical to each other.

A further description as to the "mechanism" by which the amplification occurs is given below with reference to the drawings.

The digestion resistant regions of the first and second primers may comprise at least one modified nucleotide resistant to digestion by the 5' double stranded specific exonuclease. It is a requirement that the digestion resistant region is not wholly digested by the 5' double stranded specific exonuclease. This may be achieved by appropriate selection of the type and number of modified nucleotides provided in digestion resistant region. It will generally be preferred that the region incorporate several (e.g. at least 4) modified nucleotides to ensure that the region is not totally digested. The modified nucleotides may for example be thiolated or boronated nucleotides or indeed be ribonucleotides. It is preferred that the first and second primers include at least one (e.g. preferably four and more preferably six) internal phosphorothioate linkages.

It is preferred that each of the first and second primers has 30 to 60 bases and that the third and fourth primers have about half the number (e.g. 12 to 30) of bases as the first and second primers. Each of the third and fourth primers will for preference be of a sequence corresponding to those regions of the first and second primers respectively which are 5' to the 5' end of the digestion resistant regions thereof. However we do not preclude the possibility of the 3' ends of the third and fourth primers incorporating bases corresponding to some of the modified nucleotides provided at the 5' ends of the digestion resistant region (of the first and second primers) to the extent that these modified nucleotides are likely to be digested during partial digestion of the region.

It is particularly preferred that the polymerase which is used is one which has activity at the same temperature as that at which the 5' specific double strand specific exonuclease has activity whereby the reaction may be effected isothermally. For preference this temperature is 37°C.

The 5' double strand specific exonuclease may for example be T7 gene 6 exonuclease.

The strand displacing polymerase may for example be at least one of 9°N polymerase, Klenow (exo$^-$) polymerase, *Bst* polymerase, Vent (exo$^-$) polymerase, Deep Vent (exo$^-$) polymerase, *Pfu* (exo$^-$), *Tth, Tfl, Taq* or *Bca* (exo$^-$) polymerase.

The nucleotides as identified above as (d) of the reaction mixture may be dATP, dCTP, dGTP and dTTP. It is however possible for at least a portion of at least one of these nucleotides to be replaced by a substituted nucleotide whereby a strand (generated during the reaction) incorporating the modified nucleotide is resistant to digestion by the 5' double strand specific exonuclease.

It is an advantage of the invention that the reaction may be effected without pre-manipulation (e.g. restriction digestion) of the target to be amplified, such manipulations complicating other methods known in the art. It is further advantage that non-amplifiable sequences, which might otherwise adversely affect the reaction (e.g. non-target nucleic acid, non-protected primer dimers), are degraded during the amplification process.

The invention will be further described by way of example only with reference to the accompanying drawings, in which:

Fig. 1 schematically illustrates a DNA molecule for amplification in accordance with the method of the invention;

Fig. 2 schematically illustrates four oligonucleotide sequences (primers) for use in forming an amplified product from the molecule shown in Fig. 1;

Fig. 3 schematically illustrates one embodiment of the method of the invention;

Fig. 4 schematically illustrates a second embodiment of the method of the invention; and

Figs. 5-6 illustrate the results of the examples.

In the following description, sequences which are complementary to each other are denoted by the same letters (or sequence of letters) but with one of the two complementary sequences additionally being denoted by the "prime" suffix ('). Thus X' is the complementary sequence to X.

Fig. 1 illustrates a double stranded molecule incorporating a sequence X and its complement X' to be amplified by the method of the invention.

For convenience in the following description, the sequence X is shown as having a 3' binding region denoted by D'C'. Furthermore, sequence X' is shown as having a 3' binding region B'A' (adopting the convention that a segment of the nucleic acid molecule is annotated from the 5' end of the molecule). Binding sequences D'C' and B'A' may for example each comprise 30-60 bases of which sequences A and C may each provide about half of the number of these bases. Thus for example sequences A and C may each provide 12-30 bases

Referring now to Fig. 2, there is illustrated therein four primers which are to be used in the method to be described more fully below with reference to Fig. 3. More particularly, the primers shown in Fig. 2 comprise first and second primers AB and CD respectively (also designated herein as "long" primers) together with third and fourth primers A and C respectively (also designated herein as "short" primers).

The first primer AB is capable of hybridising to the 3' binding region B'A' associated with sequence X' to be amplified. Similarly the second primer CD is capable of hybridising to the 3' binding region D'C' of the sequence X to be amplified. As such, each of primers AB and CD may comprise 30 to 60 bases. Furthermore

primer AB incorporates a digestion resistant region (denoted by the thickened line) which is comprised of at least one and preferably a plurality of modified nucleotides so as to be resistant to digestion by a 5' double strand specific exonuclease used in the reaction described more fully below and which is located between sequences A and B. Similarly the primer CD also includes a digestion resistant region between sequences C and D.

The third and fourth primers each comprise sequences A and C respectively and do not incorporate modified nucleotides.

Reference is now made to the reaction depicted in Fig. 3 which takes place in the presence of the strands to be amplified, nucleotides, a strand displacing polymerase, a 5' double strand specific exonuclease and appropriate buffers.

For simplicity, Fig. 3 does not show any hybridisation/extension reactions which would result in production of a double stranded nucleic molecule not incorporating a digestion resistant region since such double stranded molecules would be digested by the 5' double strand specific exonuclease and not contribute to the overall result of the reaction.

To effect the reaction shown in Fig. 3, the double stranded target molecule shown in Fig. 1 is manipulated, by methods known in the art, so as to be in single stranded form as depicted in Fig. 3a. In this form, the first primer AB hybridises to the binding region B'A' and primer CD hybridises to binding region D'C'. Extension of primers AB and CD by the polymerase generates the two partial double stranded products illustrated in Fig. 3b. The double stranded molecules are the converted to the products of step (d) by one of two mechanisms.

In one mechanism the double stranded regions of the products serve as substrate for the 5' double strand specific exonuclease which removes those regions

not protected by upstream (5') digestion resistant region, generating the single strand products shown in Fig.3.c.

Long primers then hybridise to their complementary sequences in the single stranded products of step (c) (Fig.3.d.).

The other, and possibly more likely, mechanism is similar to that shown below with reference to steps (g) and (h). More particularly, the 5'-ends of the extension products the long primers AB and CD are digested by the exonuclease to reveal sites to which the appropriate short primers A and C may bind. These short primers then undergo strand displacing polymerisation resulting in the production of single stranded molecules to which the long primers AB and CD as appropriate may bind (i.e. resulting in the products of step (d)).

The hybridised long primers in the products of step (d) are extended by the above polymerase to generate the two partially double stranded products shown in Fig.3.e. In these products the only region that is susceptible to attack by exonuclease are the unprotected 5' regions of the extended Long Primers. These regions are, thus, removed by action of the exonuclease to generate the products seen in Fig.3.f.

The single strand 3' overhanging regions created by the action of the exonuclease contain hybridisation sites for the short primers. Fig. 3.g. illustrates the hybridisation of Short Primers at these sites. The Short Primers are so positioned as to allow their extension by a 'strand displacing' DNA polymerase with the concurrent displacement of the downstream target copy into solution, as shown in Fig.3.h. The extended strand produced by strand displacing polymerisation of the Short Primer is not protected by nucleotide modification and will be degraded by the action of exonuclease to leave a single stranded product to which the appropriate Long Primer can adhere. Simultaneously, the displaced single strands contain, at their 3' ends, sites for hybridisation of the 3' ends of yet further Long Primers.

The four molecules, which result from strand displacing polymerisation, exonuclease degradation of susceptible double stranded regions, and hybridisation of suitable primers, is shown in Fig. 3.i.. Two of these molecules, those produced by exonuclease degradation of the unprotected Short Primer extension products, are functionally identical to those generated in Fig.3.d. and, in suitable reaction conditions, will continue to cycle through the steps described between Fig.3.d. and Fig.3.i with the production of the two extra products seen in Fig. 3.i., at each cycle, and will not be considered further in the description. The two 'extra' products shown in Fig.3.i. are further processed, firstly, by the action of DNA polymerase, which will generate double stranded molecules. The long Primers from which polymerisation occurred are partially susceptible to exonuclease degradation once they are rendered double stranded. The results of extension and exonuclease digestion of the susceptible regions are shown in Fig.3.j. As before, the digestion of the Long Primer by exonuclease reveals a site at which the short Primer will hybridise, generating the molecules seen in Fig.3.k.. Strand Displacing Polymerisation of the Short Primers (Fig. 3.l.), followed by exonuclease digestion of the extended strand, will again generate four molecules to which the Long Primer can hybridise, as shown in Fig.3.m.

The first and fourth products shown in Fig.3.m. are functionally identical to the products shown in Fig.3.i. and, in the continuance of suitable reaction conditions, will cycle through the steps described between Fig.3.i. and Fig.3.m. with the production of the two extra products seen in Fig. 3.m., at each cycle, and will not be considered further in the description.

The second and third products shown in Fig.3.m. are similar to those shown in Fig.3.d. except the molecule to which the long Primer is hybridised is bounded, at both ends, within the region to be amplified. Extension of the Long Primer by polymerase and exonuclease digestion of the susceptible region double strand produced generates the products shown in Fig.3.n.. which are functionally identical to

those shown in Fig.3.j., and, in the continuance of suitable reaction conditions, will cycle through the steps described between Fig.3.j. and Fig.3.n, with the production of the two extra products seen in Fig. 3.m., at each cycle.

A number of modifications may be made to the process illustrated in Fig 3. For example, the 5' digestible regions of the first and second primers may be identical and such that they do not hybridise to the original target molecule (whereby the first and second primers only partially hybridise thereto). In this case, the first and second primers give rise to extension products having identical sequences of their 3' ends this permitting the third and fourth primers to be identical. There is the additional advantage that the use of a short sequence (in the first and second primer) for hybridising to the original target molecule provides for higher stringency.

Alternatively or additionally the 5'-ends of the first and second primers may have a partial degree of resistance to digestion (whilst still leaving a "digestible" region of the primer) to slow down the rate at which the primer is digested in the event that the 5'-double strand specific exonuclease also has activity for digesting single strands. Clearly however this 5'-ends of these primers should not be totally resistant to digestion. The partial degree of resistance may be provided by one or more nucleotides.

Figure 4. illustrates a second embodiment of the method, which differs from the method outlined in Figure 3. by the inclusion in the reaction of modified nucleosides, which, upon incorporation into an extension product, provide some protection of downstream sequences from attack by double-strand specific 5' exonuclease.

Fig. 4.a. shows the target molecule of Fig.2. rendered single stranded, with the Long Primers of Fig. 1. hybridised thereto. Fig. 4.b. shows the product of DNA polymerase mediated extension from the hybridised primers, using the target molecule

as template. The thickened lines in Fig. 4. illustrate those regions which contain modified nucleotides and are, therefore, refractant to digestion by the double-strand specific 5' exonuclease.

The next figure (Fig.4.c.) illustrates the products of exonuclease digestion of the extended products of Fig. 4.b.. Exonuclease digestion reveals sites, on the newly synthesised strand, for the primers of the opposite pair to that used in its creation. Fig. 4.d. illustrates the product of Long Primer hybridisation at these sites.

Fig.4.e. illustrates the products of extension of the newly hybridised primers by the DNA polymerase using the synthesised target copy as template and incorporating nucleotides so modified as to render downstream (3') sequences resistant to exonuclease attack.

Fig. 4.f. illustrates the products of the action of double-strand specific 5' exonuclease on the susceptible regions of the products shown in Fig. 4.e. Such action reveals hybridisation sites for the Short Primers of Fig. 2., and Fig. 4.g. shows the products of hybridisation of the short primers onto these.

Fig. 4. h. illustrates the extension of the attached Short Primer sequences, using the target copy as template, by the 'strand displacing' polymerase, with concomitant displacement of the downstream sequence into solution. The extending Short Primer incorporates the modified nucleotides such that the newly synthesised region is refractant to the action of double-strand specific 5' exonuclease. The products of the strand displacing polymerisation are, therefore, resistant to exonuclease attack, except for the unprotected sequence of the Short Primer itself, which will therefore be removed. Removal of the Short Primer sequence will regenerate the site for Short Primer hybridisation. The second and third products of Fig. 4.i. illustrate the products of Short Primer hybridisation to these exposed sequences. It can be seen that these products are functionally identical to the products shown in Fig.4.g. and, in the

continuance of suitable reaction conditions, will cycle through the steps described between Fig.4.g. and Fig.4.i. with the production of the two extra products seen in Fig. 4.i., at each cycle. These products will not, therefore, be considered further in the description.

The first and fourth products shown in Fig.4.i. illustrate the single stranded displacement products of Fig. 4.h. with, hybridised at their 3' ends, the 3' ends of Long Primer sequences. Extension from these sequences, incorporating 'modified' nucleotides, and exonuclease digestion of vulnerable regions of the double stranded products of such extension, will yield the products shown in Fig. 4.j.. These products have short, single stranded, regions at one end, which are complementary to, and hence hybridisation sites for, the Short Primers.

Fig. 4.k. illustrates hybridisation of Short primers at their hybridisation sites.

Fig. 4.l. illustrates strand displacing polymerisation of the hybridised Short Primer sequences, using the target copy as template, incorporating modified nucleotides to protect newly synthesised sequences from exonuclease digestion.

Fig. 4.m. illustrates the products of the strand displacing polymerisation shown in Fig. 4.l. following exonuclease digestion of susceptible regions and hybridisation of primers at potential hybridisation sites. The displaced single strands (products one and four shown) contain sites for hybridisation of Long Primers and are then functionally identical to the products shown in Fig 4.i.. In the continuance of suitable reaction conditions, these products will cycle through the steps described between Fig.4.i. and Fig.4.m. with the production of the two extra products seen in Fig. 4.m., at each cycle.

Likewise, the other two products in Fig. 4.m. (the second and third products) are functionally identical to the products shown in Fig. 4.j. and, in suitable reaction

conditions, will continue to cycle through the steps described between Fig.4.j. and Fig.4.m. with the production of the two extra products seen in Fig. 4.m., at each cycle.

For reasons of simplicity the methods outlined have been described in terms of discrete steps involving the actions of the of the enzymes involved running to completion before the next step in the process begins. While, in certain circumstances, this may be possible, by manipulation of conditions during the reaction such that only one or other of the enzymes involved is active at any given point in the reaction, in the preferred embodiment of the reaction it is envisaged that the reaction will occur in conditions which promote the simultaneous action of all components of the reaction.

It should be further noted that, in a number of the schematic steps, when hybridisation sites are produced, since Long and Short primers share sequence homology, either may come to occupy those sites and, furthermore, may not be able to take part in the reaction as described. In these cases processes other than those explicitly described may occur. These might include;

a) Short Primer occupying the hybridisation sites outlined in Fig3.a. d. i. (the second and third products) and m. (the second and third products). In these cases, the likely outcome is a fatuous extension of the primer, using the target copy as template, to give an unprotected extension copy, which will be removed, by the action of the exonuclease, to reveal the hybridisation site once more.

b) Short Primer occupying the hybridisation sites outlined in Fig. 4. a. and d.. In these cases the likely outcome is extension of the primer, using the target copy as template, and incorporating modified nucleotides such that the newly synthesised material is protected from digestion by the exonuclease. This product differs from the products described in the relevant figures only in the extent of modification of the sequence 3' to the short primer, which the Long Primer would have occupied. This

should have little or no effect upon amplification and these products should become incorporated into the reaction in the same way as Long Primer products would.

c) Long Primer occupying the hybridisation sites outlined in Fig 3. g. and k. and Fig 4. g. i. (the second and third products) k. and m. (the second and third products). In these cases, the most likely outcome is the digestion of the unprotected 5' region of the Long Primer, which will cause it to dissociate from the hybridisation site, revealing the site once more.

While the above reactions may affect the efficiency of the amplification reaction, they do not alter the nature of the processes involved and will not be discussed further.

For the reasons outlined above, the schematic diagrams in Fig. 3. and Fig. 4. should be taken as indicative of the processes occurring, rather than an exact blueprint of the reaction.

Additional modifications/additions to the method may be made using techniques known in the art. These may include;

a) Using a non-natural target sequence such as a ligation product, a wholly synthesised sequence, a closed circular target molecule etc.

b) Using as a target RNA, either naturally derived or wholly, or partially, manufactured, which can be converted to a homologous or complementary DNA sequence by methods known in the art.

c) Using Long Primers which share 5' sequence homology such that a single Short Primer could serve to generate product from each end of the target molecule during the amplification reaction.

d) Using target sequences or one, or more, primers which are covalently, or non-covalently, attached to a solid phase.

e) While the example reactions below are performed at 37°C using T7 gene 6 exonuclease and the exonuclease deficient variant of the Klenow fragment of E. Coli DNA polymerase, there are polymerase enzymes known in the art which have higher temperature optima. In the presence of a thermophilic double strand specific 5' exonuclease, the reaction should occur at elevated temperatures.

The invention will be illustrated by the following non-limiting examples.

Example 1

An amplification reaction was carried out using the following primers, template and reaction conditions. Two sets of primers were used. The first were primers EDA M1 and EDA M2 (a 37-mer and a 38-mer) containing four internal phosphorothioate linkages in series. The second set were the short displacement primers (16-mers) EDA D1 and EDA D2 whose sequences were identical to the first 16 bp of the long primers reading from the 5' end.

EDA M1 (SEQ ID NO. 1)

5' TGG TGT GTG GAT CAA CGG CGC *ACC C*TA GAG GTC TTC A 3'

EDA M2 (SEQ ID NO. 2)

5' ACA TCA CCC ATG AAA CGC GCG *GCA A*TC GGT TTG TTG TA 3'

(Italicised bases indicate the location of the phosphorothioate linkages.)

EDA D1 (SEQ ID NO. 3)

5' TGG TGT GTG GAT CAA C 3'

EDA D2 (SEQ ID NO. 4)

5′ ACA TCA CCC ATG AAA C 3′

The short primers were used to amplify a conserved 129bp region of the glycoprotein B gene of Human CMV by PCR. This was the template used in the Amplification reaction and due to the primers used, the PCR amplified fragment contained no phosphorothioate linkages 21bp or other means of protection.

CMV 129bp fragment sequence (SEQ ID NO. 5)

3′ TGG TGT GTG GAT CAA CGG CGC ACC CTA GAG GTC TTC CAA GGA ACT CAG CAA GAT CAA CCC GTC AGC CAT TCT CTC GGC CAT TTA CAA CAA ACC GAT TGC CGC GCG TTT CAT GGG TGA TGT 5′

The following reaction mixture was prepared in a 0.2 ml thin walled PCR tube:5µl of 10x Klenow (exo$^-$) reaction buffer (500 mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 10 mM DTT, 0.5 mg/ml BSA [Amersham]), 2µl of 10mM dNTP's (Pharmacia), 150 pmoles each of primers EDA M1 and M2, approximately 120 pmoles of 129 bp template and ultrapure water to a final volume of 50µl. Samples were mixed and incubated at 96°C for three minutes to denature and then allowed to cool to 37°C for 1 minute before addition of 20 units of Klenow (exo-) polymerase. Incubation was allowed to proceed at 37°C for two minutes before addition of the following mixture: 120 pmol each of primers EDA D1 and D2, 15 units of T7 gene 6 exonuclease, 5µl of 10x Klenow (exo$^-$) polymerase (Amersham), and ultrapure water to a final reaction volume of 50µl. 16µl aliquots were taken at the following intervals '0 time', 60, 90, 120, 150 and 180 minutes. These were mixed with an appropriate dilution of loading dye and stored at 4°C until ready for visualisation by electrophoresis on a 3% agarose gel containing ethidium bromide to a final concentration of 0.3µg/ml.

The results are shown in Fig. 5 in which lane is a 100bp marker and lanes 2-7 respectively illustrate the results obtained at "0 time" 60, 90, 120, 150 and 180 minutes. The production of amplified product is clearly shown in lanes 5-7.

Example 2

An amplification reaction was carried out using the following primers, template, and reaction conditions. Two sets of primers were used. The first were long primers EDLM and EDUM (both 35mers) containing six internal phosphorothioate linkages in series. The second set were the short displacement primers (16-mers) EDUDP and EDLDP whose sequences were identical to the first 16 bp of the long primers and which contained two phosphorothioate linkages at their 5' end.

EDLM (SEQ ID NO. 6)

5' CGT TCA TCC ATA GTT GCC *TGA CTC* CCC GTC GTG TA 3'

'EDUM (SEQ ID NO. 7)

5' TAT TGC TGA TAA ATC TGG *AGC CGG* TGA GCG TGG GT 3'

EDUDP (SEQ ID NO. 8)

5' *CG*T TCA TCC ATA GTT G3'

EDLDP (SEQ ID NO. 9)

5' *TA*T TGC TGA TAA ATC T 3'

EDA003B (SEQ ID NO. 10)

5'CCC TCC CGT ATC GTA GTT AT 3'

EDA001MO (SEQ ID NO. 11)

5' GTA TCA TTG CAG CAC TGG 3'

(Italiscised bases indicate the location of the phosphorothioate linkages. Bases in bold indicate the presence of biotin label. Primers EDUDP (batch 1139-1) and EDLDP (batch 1139-2) were supplied by MWG-Biotech UK, Milton Keynes. UK; primers EDLM and EDUM were synthesised at Tepnel Life Sciences UK).

The long primers were used to amplify a 129bp region (between bases 1568 and 1662) of pUC 19 by PCR. This was the template used in the EDA reaction and due to the primers used, the PCR amplified fragment contained phosphorothioate linkages 18 bp from the 5' terminus of both strands.

The following master mix was prepared: 15ml of x10 Klenow (exo-) reaction buffer;, 12ml of 10mM dNTP's; 6ml of 20 pmole/ml EDUM; 6ml of 20 pmole/ml EDUM; and ultrapure water to 120ml final volume. 10ml of ultrapure water containing 25ng, 1ng or 0ng of PCR amplified 129 bp pUC 19 template, was added to 40ml of the above master mix, in 0.2 ml thin walled PCR tubes These samples were heated to 98°C for 2 minutes on a Biometra Trio 48 thermocycler before addition of 50ml of a master mix comprising; 50ml of x10 Klenow (exo-) buffer, 4.5ml of 50 pmole/ml EDLDP; 4.5ml; 50 pmole/ml EDUDP ; 4.5m of 50 pmole/ml EDUDPl; 6ml; 5unit/ml Klenow (exo-) polymerase; 1.8ml of 50 units/ml T7 gene6 exonuclease; and ultrapure water to 150µl final volume. .

Samples were incubated at 37°C for 4 hours. 17µl aliquots were visualised on a visualised by electrophoresis on a 3% agarose gel containing ethidium bromide to a final concentration of 0.3µg/ml (Figure 6a). In Fig 6a, lane 1 is a 100 bp marker. and lanes 2-4 show the results obtained using 25ng, 1ng and 0ng of template respectively. The production of amplified product can be seen in lanes 2 and 3.

In addition, the products from the reactions containing 1 ng of template and 0 ng of template were tested using the DARAS™ system to confirm that the fragments generated were of the correct sequence. The oligonucleotide EDA001MO was covalently attached to a solid phase and used to capture the amplified product. Once captured the identity of the sequence was further confirmed by detection with the labelled probe EDA001B which was complementary to a sequence within the 129 bp fragment (Figure 6b).

CLAIMS

1. A method of amplifying complementary first and second nucleic acid sequences each of which has a binding region at its 3' end, the method comprising treating the separated single stranded sequences with

(a) first and second primers each capable of hybridising to the 3'-binding regions of the first and second strands respectively and each including remote from its 5'-end a digestion resistant region which, with the primer hybridized to the complementary 3'-binding region, allows only partial digestion of the primer by the enzyme (d) having 5'-double strand specific exonuclease activity,

(b) third and fourth primers each having a degree of sequence homology with the partially digestible regions of the first and second primers respectively whereby the third and fourth primers are capable of hybridising to the 3'-binding regions of the first and second strands respectively,

(c) an enzyme having strand displacing polymerase activity,

(d) an enzyme having 5' double stranded specific exonuclease activity, said enzyme (d) possibly being provided by enzyme (c) in the case where the latter also has the required exonuclease activity, and

(e) nucleoside triphosphates,

under conditions permitting hybridisation, exonuclease digestion and strand displacement polymerisation thereby producing an amplified amount of the first and second strands.

2. A method as claimed in claim 1 wherein the double stranded nucleic acid molecule is generated *in situ* from a single stranded nucleic acid molecule.

3. A method as claimed in claim 1 or 2 wherein the digestion resistant region is provided by modified nucleotides or ribonucleotides.

4. A method as claimed in claim 3 wherein the modified nucleotides provide phosphorothiate linkages which provide the resistance to digestion by the exonuclease.

5. A method as claimed in any one of claims 1 to 4 wherein the first and second primers each comprise 30 to 60 bases.

6. A method as claimed in claim 5 wherein the digestion resistant region is provided 15 to 25 bases from the 5' end of the first and second primers.

7. A method as claimed in any one of claims 1 to 6 wherein the third primer is of a sequence corresponding to at least a portion of the sequence in the first primer on the 5' side of the digestion resistant region of that primer.

8. A method as claimed in any one of claims 1 to 7 wherein the fourth primer is of a sequence corresponding to at least a portion of the sequence in the second primer on the 5' side of the digestion resistant region of that primer.

9. A method as claimed in any one of claims 1 to 8 wherein the third and fourth primers comprise 12 to 30 bases.

10. A method as claimed in any one of claims 1 to 9 wherein the 5' double strand specific exonuclease is T7 Gene 6 exonuclease.

11. A method as claimed in any one of claims 1 to 10 wherein the strand displacing DNA polymerase is at least one of, 9°N polymerase, Klenow (exo$^-$) polymerase, *Bst* polymerase, Vent (exo$^-$) polymerase, or Deep Vent (exo$^-$) polymerase, *Pfu* (exo$^-$) polymerase, *Tth* polymerase, *Tfl* polymerase, *Taq* polymerase or *Bca* (exo$^-$) polymerase.

12. A method as claimed in any one of claims 1 to 11 wherein the steps of exonuclease digestion and strand displacing polymerisation are effectively separated by performing the two reactions separately by removal of enzyme between steps, or, under conditions which favour the action of one or other enzyme.

13. A method as claimed in any one of claim 1 to 12 effected isothermally.

14. A method as claimed in anyone of claims 1 to 13 wherein the digestible regions of the first and second primers are of identical sequence and the third and fourth primers are identical to these sequences.

15. A method as claimed in anyone of claims 1 to 14 wherein the 5'-ends of the first, second, third and fourth primers have a partial degree of resistance to digestion.

16. A method as claimed in any one of claims 1 to 15 wherein the amplification occurs in the presence of further primers specific to other target sequences (multiplex amplification) or to all or some of the same target sequence (nested amplification).

17. A method as claimed in any one of claims 1 to 16 wherein at least a portion of at least one of the nucleoside triphosphates provided as (e) of claim 1 is/are a modified such that when it is incorporated in a growing nucleic acid chain it is resistant to digestion by the exonuclease.

18. A method as claimed in any one of claims 1 to 17 wherein the nucleic acid is DNA.

19. A method of amplifying complementary first and second nucleic acid sequences each of which has a binding region at its 3' end, the method comprising the steps of

(i) forming a reaction mixture comprised of the separated single strands together with

(a) first and second primers each capable of hybridising to the 3'-binding regions of the first and second strands respectively and each including remote from its 5'-end a digestion resistant region which, with the primer hybridised to the complementary 3'-binding region, allows only partial digestion of the primer by the enzyme (d) having 5'-double strand specific exonuclease activity,

(b) third and fourth primers each having a degree of sequence homology with the partially digestible regions of the first and second primers respectively whereby the third and fourth primers are capable of hybridising to the 3'-binding regions of the first and second strands respectively,

(c) an enzyme having strand displacing polymerase activity,

(d) an enzyme having 5' double stranded specific exonuclease activity, said enzyme (d) possibly being provided by enzyme (c) in the case where the latter also has the required exonuclease activity, and

(e) nucleoside triphosphates.

(ii) effecting a reaction under conditions permitting hybridisation, exonuclease digestion and strand displacement polymerisation thereby producing an amplified amount of the first and second strands.

20. A method of amplifying complementary first and second nucleic acid sequences each of which has a binding region at its 3' end, the method comprising treating the separated single stranded sequences with

(a) first and second primers each capable of hybridising to the 3'-binding regions of the first and second strands respectively and each including remote from its 5'-end a digestion resistant region which, with the primer hybridised to a complementary, allows only partial digestion of the primer by the enzyme (d) having 5'-double strand specific exonuclease activity,

(b) third and fourth primers each having a degree of sequence homology with the particularly digestible regions of the first and second primers respectively whereby the third and fourth primers are capable of hybridising to the 3'-binding regions of the first and second strands respectively,

(c) an enzyme having strand displacing polymerase activity,

(d) an enzyme having 5' double stranded specific exonuclease activity, said enzyme (d) possibly being provided by enzyme (c) in the case where the latter also has the required exonuclease activity, and

(e) nucleoside triphosphates, at least a portion of at least one of which is modified such that when it is incorporated into a growing nucleic it is resistant to digestion by the exonuclease.

under conditions permitting hybridisation, exonuclease digestion and strand displacement polymerisation thereby producing an amplified amount of the first and second strands.

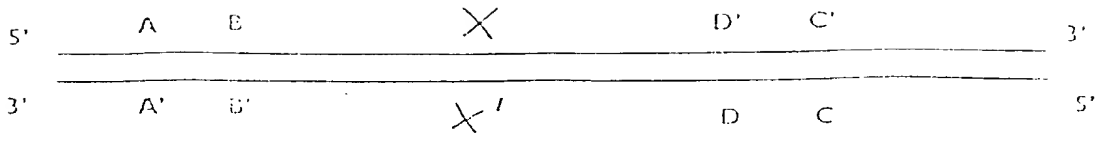


FIG. 1

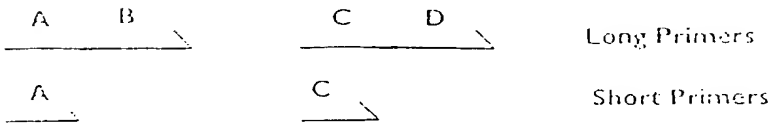


FIG. 2

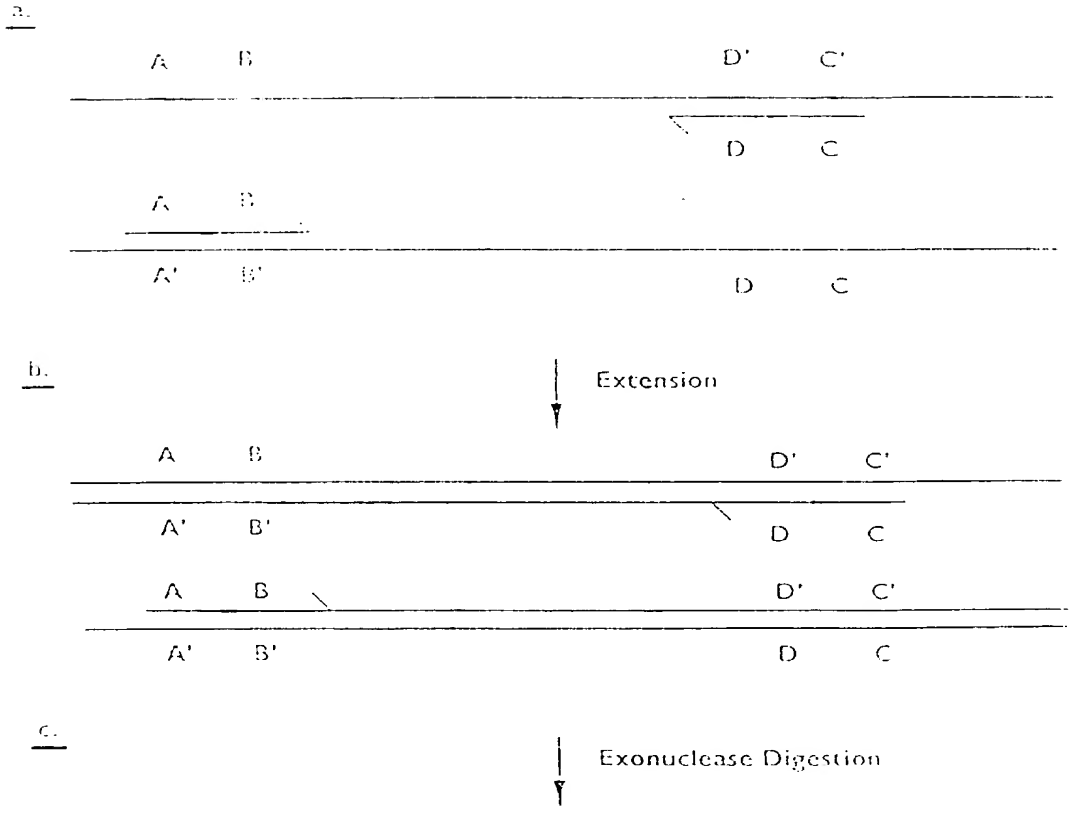

a.
A B
D' C'
D C
A B
A' B'
D C
b.
Extension
A B
D' C'
A' B'
D C
A B
D' C'
A' B'
D C
c.
Exonuclease Digestion

FIG. 3

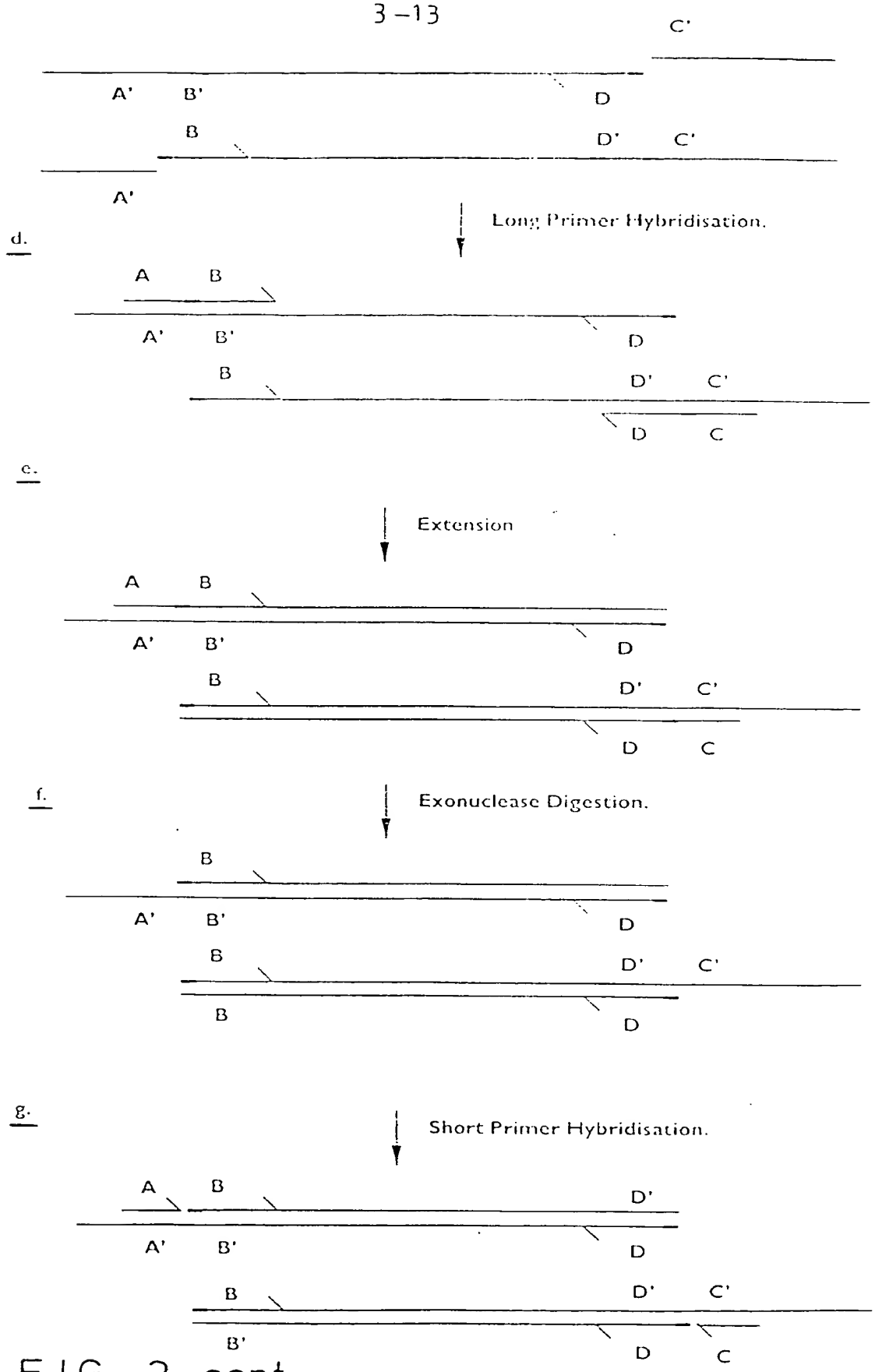

C'
A'
B'
D
B
D'
C'
A'
Long Primer Hybridisation.
d.
A
B
A'
B'
D
B
D'
C'
D
C
e.
Extension
A
B
A'
B'
D
B
D'
C'
D
C
f.
Exonuclease Digestion.
B
A'
B'
D
B
D'
C'
B
D
g.
Short Primer Hybridisation.
A
B
D'
A'
B'
D
B
D'
C'
B'
D
C

FIG. 3 cont.

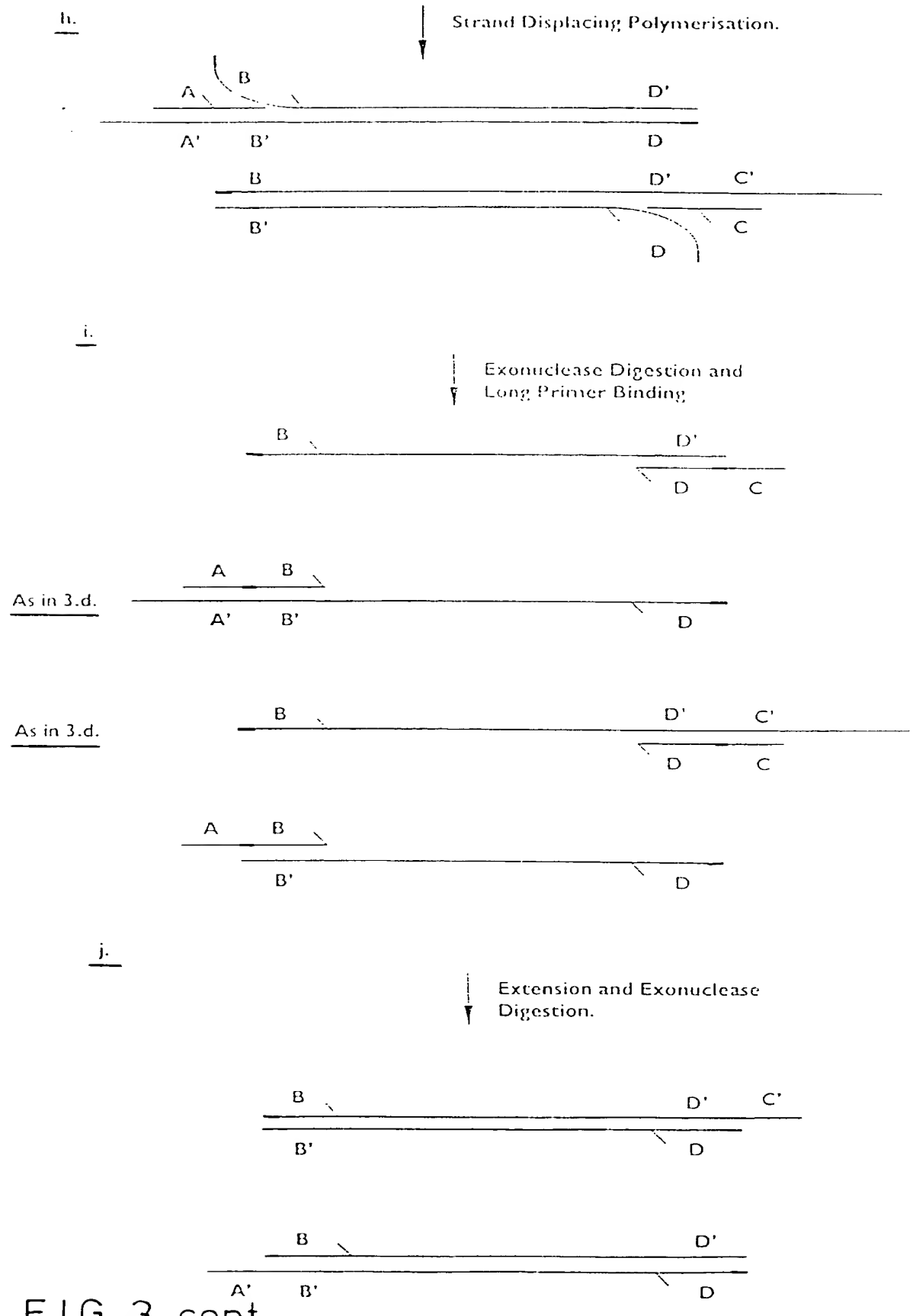
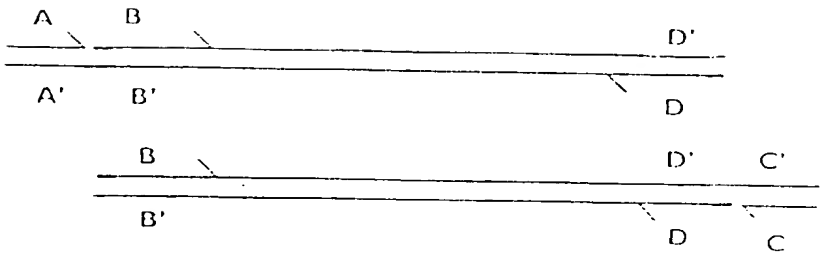

h.
Strand Displacing Polymerisation.
A
B
D'
A'
B'
D
B
D'
C'
B'
C
D
i.
Exonuclease Digestion and
Long Primer Binding
B
D'
D
C
As in 3.d.
A
B
A'
B'
D
As in 3.d.
B
D'
C'
D
C
A
B
B'
D
j.
Extension and Exonuclease
Digestion.
B
D'
C'
B'
D
B
D'
A'
B'
D

FIG. 3 cont.

5-13

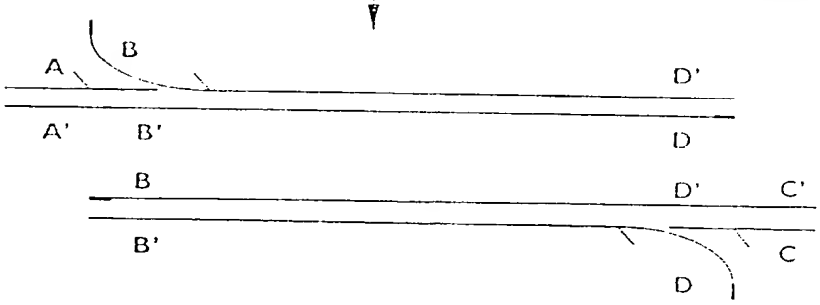
k.

Short Primer Hybridisation.



l.

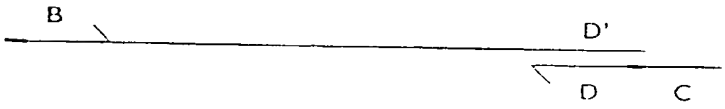
Strand Displacing Polymerisation.



m.

Exonuclease Digestion and
Long Primer Binding

As in 3.i.



As in 3.i.

FIG. 3 cont.

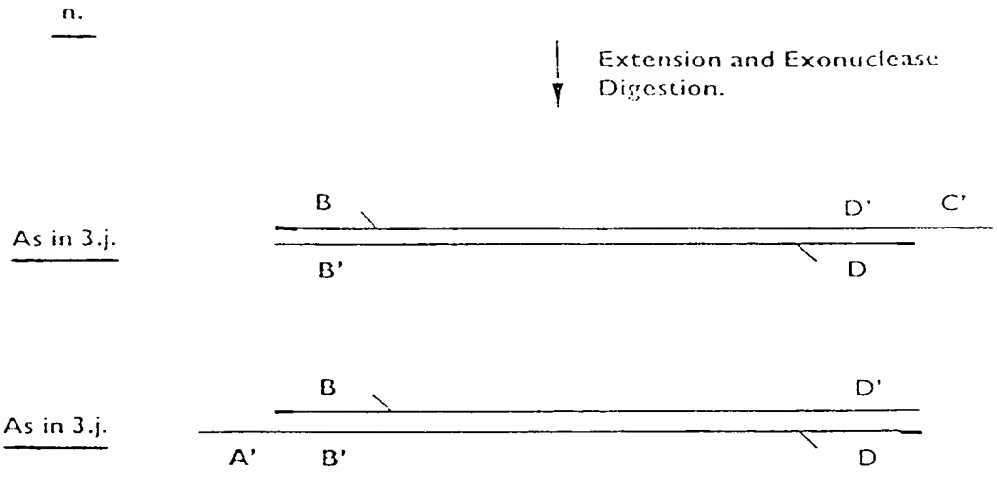

n.
Extension and Exonuclease Digestion.
As in 3.j.
B
D'
C'
B'
D
As in 3.j.
B
D'
A'
B'
D

FIG. 3 cont.

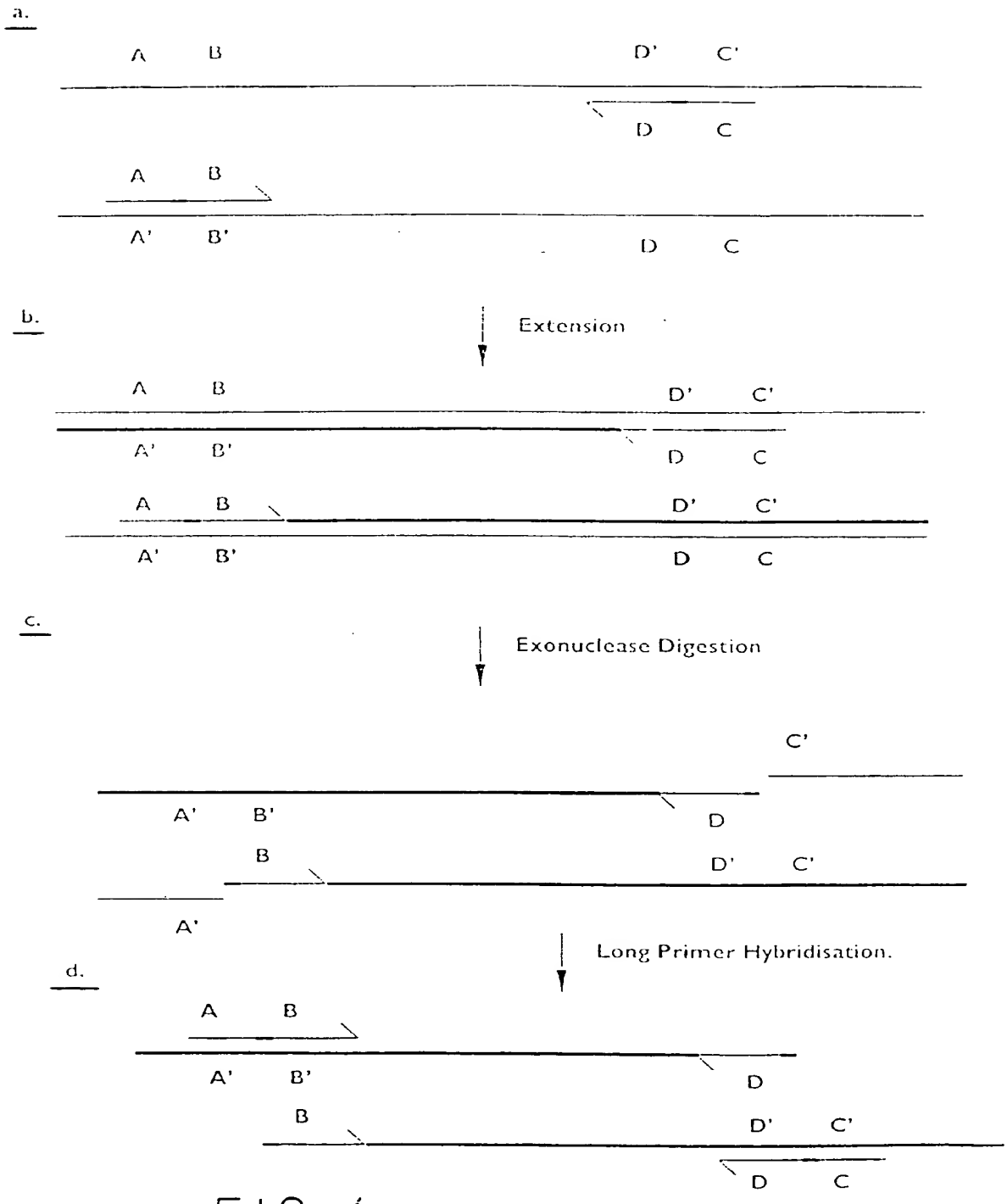

a.
A
B
D'
C'
D
C
A
B
A'
B'
D
C
b.
Extension
A
B
D'
C'
A'
B'
D
C
A
B
D'
C'
A'
B'
D
C
c.
Exonuclease Digestion
C'
A'
B'
D
B
D'
C'
A'
Long Primer Hybridisation.
d.
A
B
A'
B'
D
B
D'
C'
D
C

FIG. 4

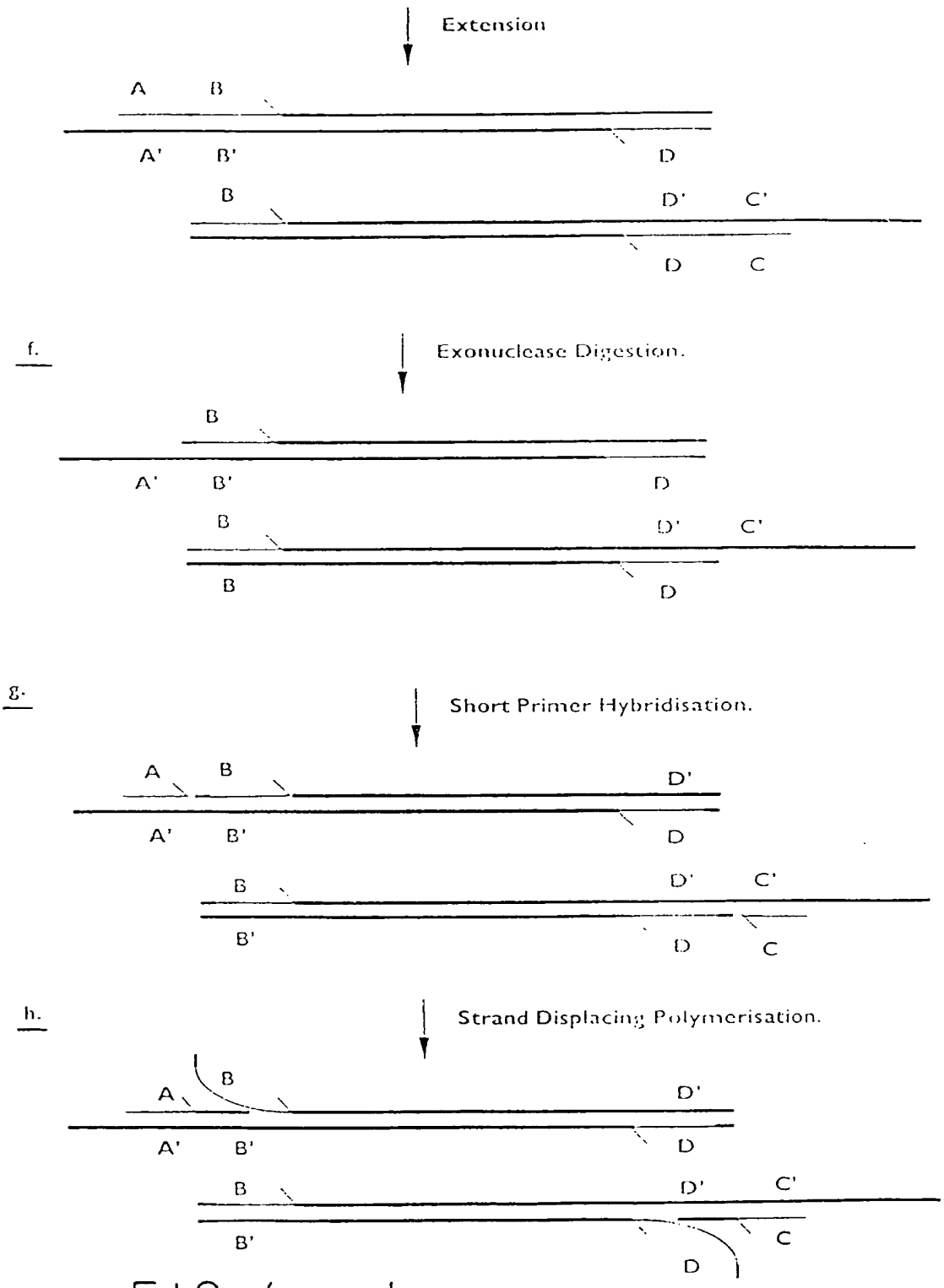

e.
Extension
A
B
A'
B'
D
B
D'
C'
D
C
f.
Exonuclease Digestion.
B
A'
B'
D
B
D'
C'
B
D
g.
Short Primer Hybridisation.
A
B
D'
A'
B'
D
B
D'
C'
B'
D
C
h.
Strand Displacing Polymerisation.
A
B
D'
A'
B'
D
B
D'
C'
B'
C
D

FIG 4 cont.

9-13

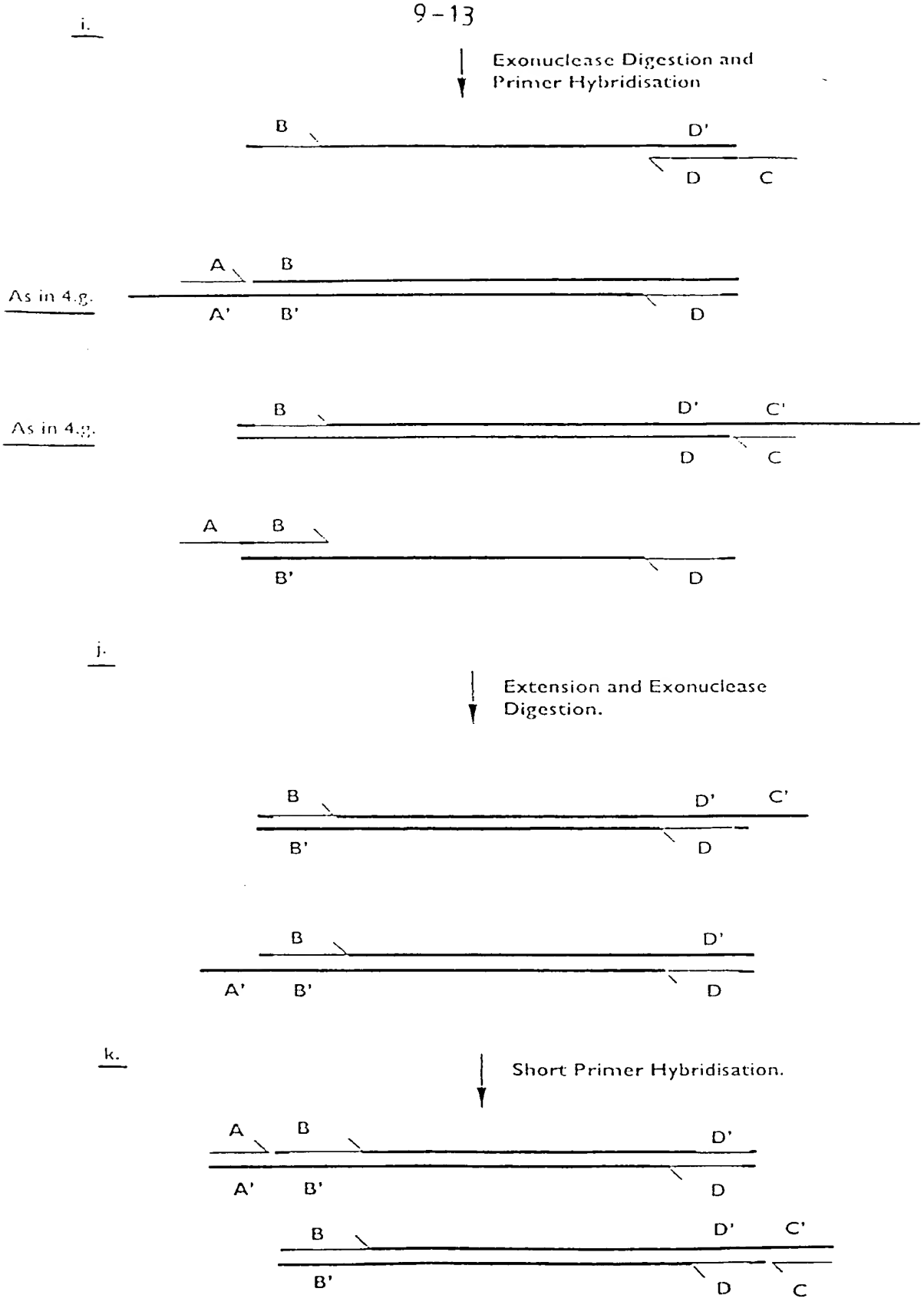


FIG. 4 cont.

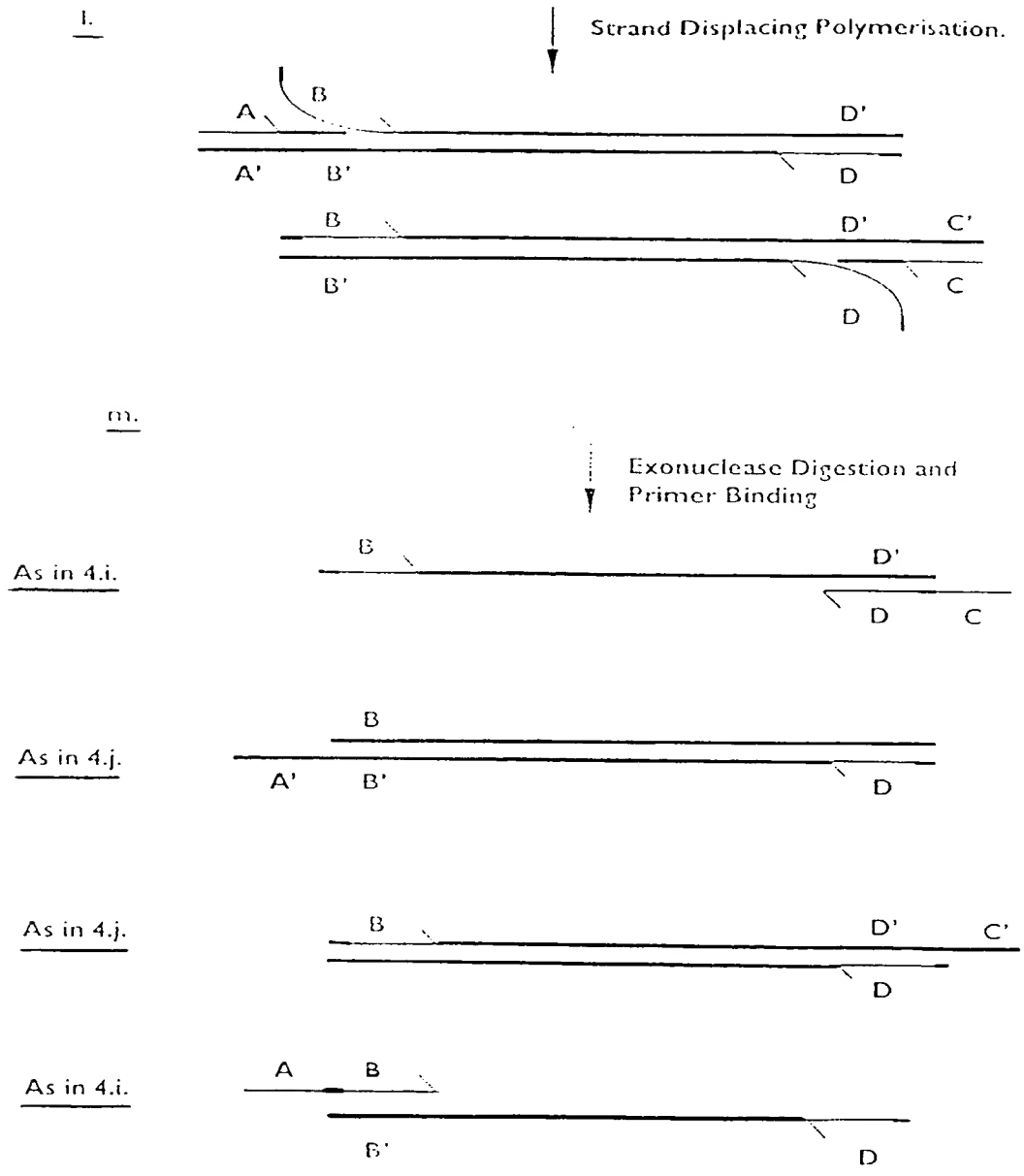

l.
Strand Displacing Polymerisation.
A
B
D'
A'
B'
D
B
D'
C'
B'
C
D
m.
Exonuclease Digestion and
Primer Binding
As in 4.i.
B
D'
D
C
As in 4.j.
B
A'
B'
D
As in 4.j.
B
D'
C'
D
As in 4.i.
A
B
B'
D

FIG. 4 cont

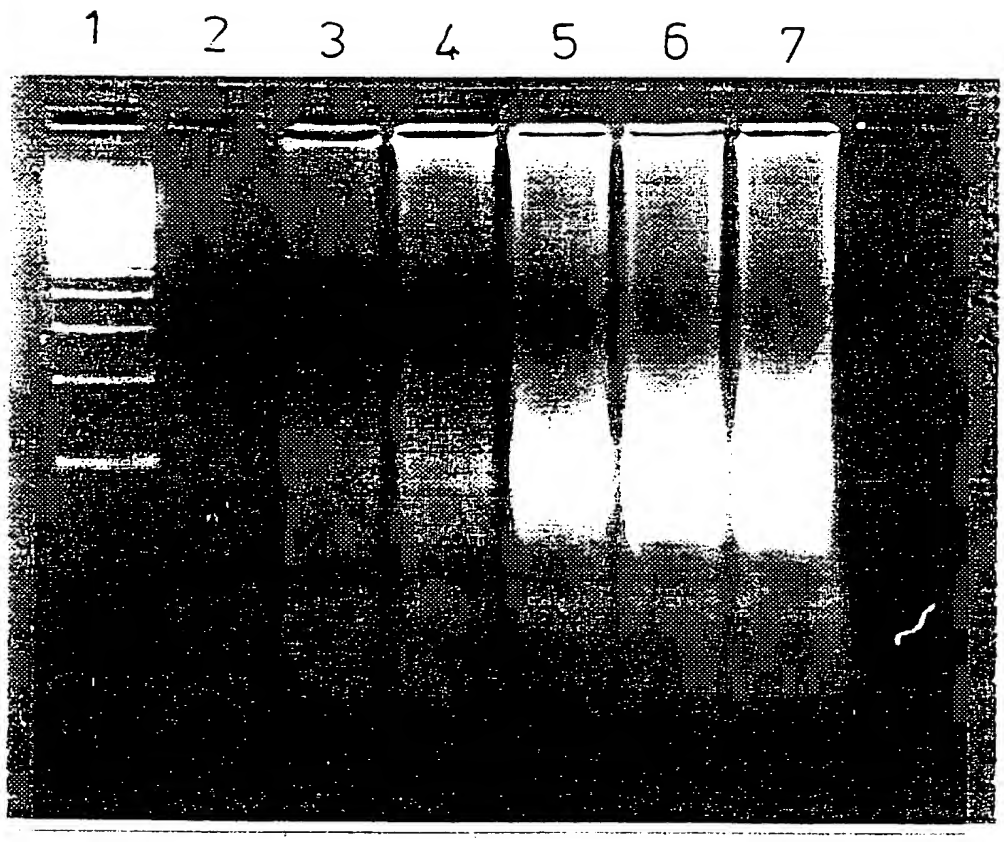


FIG. 5

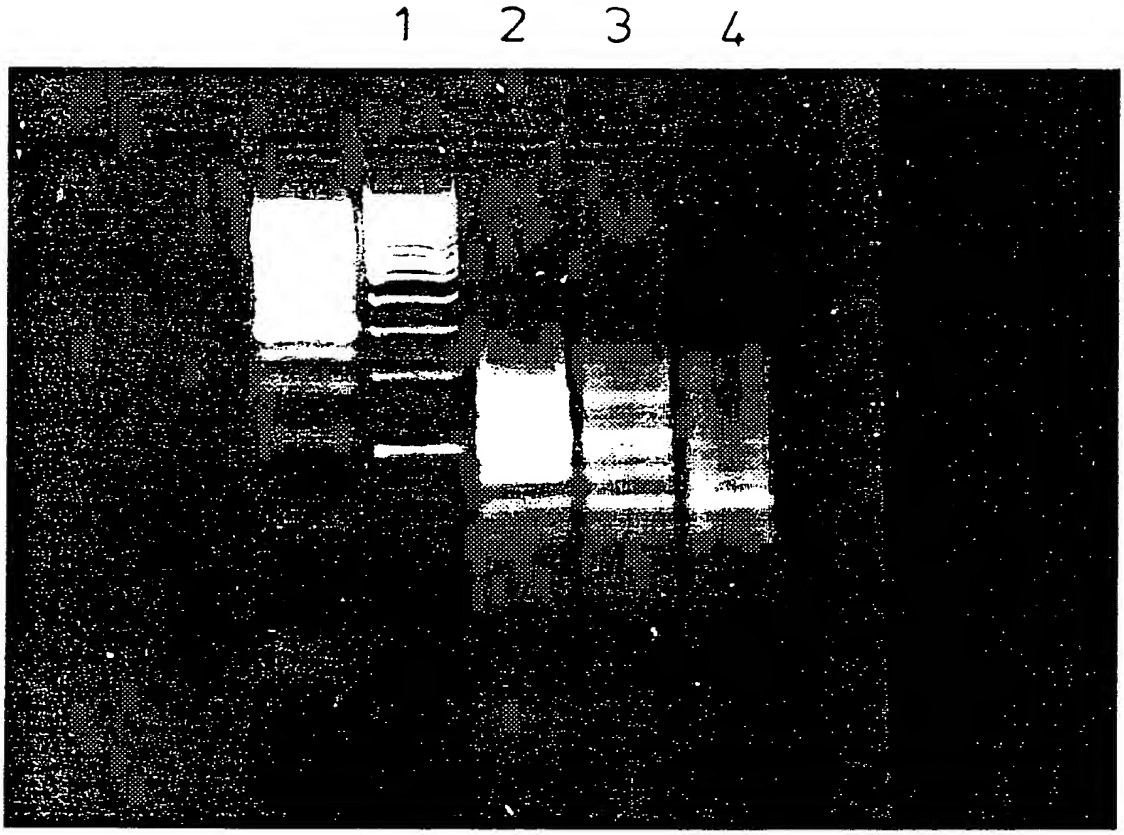


FIG 6a

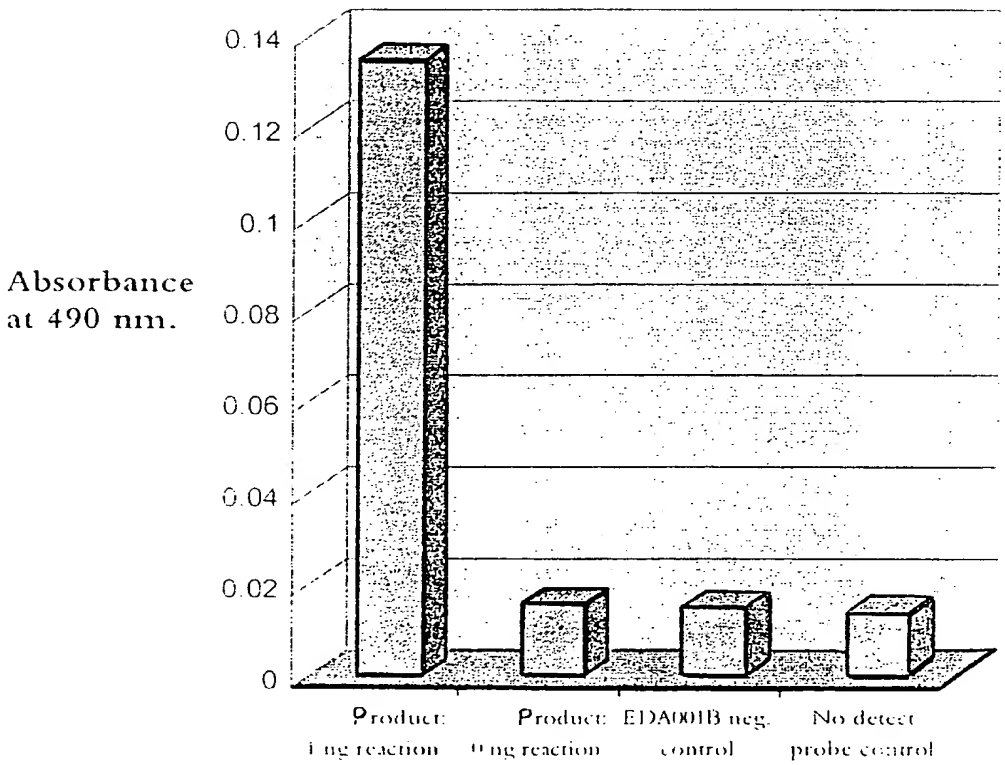

Absorbance at 490 nm.
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Product: 1 ng reaction
Product: 0 ng reaction
EDA00HB neg. control
No detect probe control
Samples and controls.

FIG. 6b

ELSEVIER

Journal of Virological Methods 72 (1998) 117–121

Journal of Virological Methods

Brief Report

Use of T7 gene 6 exonuclease and phosphorothioated primers for the manipulation of HIV-1 infectious clones

Luciana Jesus da Costa, Amilcar Tanuri *

Laboratório de Virologia Molecular, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Ilha do Fundao, Rio de Janeiro, 21943-900, Brasil

Received 2 September 1997; accepted 21 November 1997

Abstract

A method is described for the efficient substitution, deletion or insertion of any desired DNA sequence into any viral infectious clones without the limitation of naturally occurring restriction sites. The technique employs the polymerase chain reaction combined with the resistance of 2'-deoxynucleotides 5'-*O*-(1-thiotriphosphate) dNTPs [S] bonds (phosphorothiate bonds) to the 5'-3' double strand specific T7 gene 6 exonuclease (T7 Exo) digestion. Primers used to amplify the DNA target regions being manipulated present three phosphorothioate bonds from the fifteenth base at the 5' end. The enzyme activity was shown to be completely inhibited by the presence of more than one phosphorothioate residue at the 5' end of the DNA molecules. When the amplification products are submitted to the exonuclease digestion the hydrolytic T7 Exo activity generates a short single strand DNA tail which contains the nucleotide integrity of the 3' strand. Since the ends of two independently amplified products overlap they can regenerate a stable recombinant structure when further combined in the same reaction tube in the presence of T4 DNA ligase. This new method can be used for manipulating an HIV-1 full-length clone belonging to subtype D replacing the *env* (gp120) gene for an F subtype sequence.

Keywords: HIV-1 infectious clone; T7 Exo; Phosphorothioate primers

Manipulation of infectious clones of viruses by mutating, deleting or substituting defined regions has been an invaluable tool to investigate both viral regulation and gene function. Engineering such recombinants relies on the presence of restriction sites flanking the target regions, therefore limiting the constructions of specific viral clones.

* Corresponding author. Present address: Visitor Researcher at the HIV Division/AIDS Branch, CDC, Atlanta, GA 30333, USA. E-mail: act7@cdc.gov

0166-0934/98/$19.00
PII S0166-0934(98)00009-3

The purpose of this study was to develop a strategy to either substitute or delete specific regions of any DNA sequence independent of using naturally occurring restriction sites. This approach employs the polymerase chain reaction (PCR) combined with the resistance of phosphorothioate bonds to the 5′-3′ double strand specific T7 Exo digestion (Kerr and Sadowski, 1972). The enzyme activity was shown to be completely inhibited by the presence of more than one phosphorothioate residue at the 5′ end of the DNA molecules (Nikiforov et al., 1994). Primers used to amplify the DNA target regions being manipulated present three phosphorothioate bonds from the fifteenth base at the 5′ end. When the amplification products are submitted to the exonuclease digestion the hydrolytic T7 Exo activity continues straight forward through the 5′ end until it reaches the phosphorothioate bonds, generating a short single strand DNA tail which contains the nucleotide integrity of the 3′ strand. Since the ends of two independently amplified products overlap they can regenerate a stable recombinant structure when further combined in the same reaction tube in the presence of T4 DNA ligase. The overall approach is outlined in Fig. 1.

The method was illustrated in an experiment where an HIV-1 full-length clone belonging to subtype D, named Z6, carried by pBR322 (Srinivasan et al., 1987) was manipulated. The goal was to replace the *env* (gp120) gene of Z6 clone with that of the F subtype, which is carried by pENVBZ59 (a gp120 sequence of a Brazilian isolate that was cloned in a pCR2 vector; TA Cloning Kit, Invitrogen). A large 13.0-kb fragment encompassing the HIV genome, but the gp120 gene, as well as the vector backbone was amplified using pZ6 as template; the sense primer corresponds to nucleotide positions 7754–7790 of pZ6 clone (ExtF, 5′- ATAGGACTAGGAG^{S}C^{S}T^{S}ATGTTCCTTGGGTTCTTGGGA-3′) and the antisense primer corresponds to nucleotide positions 6195–6231 of pZ6 clone (ExtR, 5′-CATTGCCACTGTC^{S}T^{S}T^{S}CTCGCTCTTTCTCTTATTCTA-3′) in which S stands for (dNTP[αS]). Conditions for PCR amplification were as described in the XL PCR kit (Perkin Elmer, NJ) as followed. The reactions were carried out based on the hot start procedure, which consists of the use of a layer to separate two mixture reactions, the lower and upper reagent mix: the lower reagent mix, which is placed at the bottom of the tube, contains 12 μl of 3.3 × XL buffer, 200 μM of deoxynucleotide triphosphate, 4.4 μl of $Mg(OAc)_2$ 25 mM, 25 pmol of each primer and water in a total volume of 40 μl. One AmpliWax PCR GEM 100 was added on the top of the mixture, followed by heating the tubes at 75°C for 5 min in order to melt the wax. The reaction tubes were then cooled to room temperature. The upper reagent mix consists of 10 ng of DNA template, 18 μl of the 3.3 × XL buffer, 2.0 μl of rTh DNA polymerase and water to total volume of 60 ml. The mixture is then placed over the wax layer. The thermocycling conditions were as follows: heating at 94°C for 2 min, 16 cycles of 95°C for 15 s and 60°C for 12 min, followed by 24 cycles of 95°C for 15 s and of 68°C for 13 min with extensions of 15 s per cycle, and a final extension of 68°C for 12 min. Another fragment of 1.5 kb containing the gp120 gene of F subtype was amplified using pENVBZ59 as template. The reactions were carried out with 20 pmol of each primer (IntF: 5′-ACAGTGGCAATGASGSASGCGAGGGAG-TAGAGAGGAAT-3′) and (Ext.R: 5′-TCCTAGTCCTATTSGSCSTCTTTTTTCTCTTTCCACCAC-3′), 10 ng of pENVBZ59 as template, 200 mM of each deoxynucleotide triphosphate, 2.5 U of Taq polymerase and 2.5 mM $MgCl_2$. The reac-

Fig. 1. Schematic representation of the new strategy. (1) PCR amplification of the vector backbone (Plasmid A) and the DNA sequence to be replaced (Plasmid B; the donor sequence vector). (2) Treatment of each amplified product with Exo T7 enzyme to expose the 3′ overlapping end of each strand. (3) Enzyme inactivation with temperature treatment (70°C for 10 min). (4) T4 ligation reaction of the vector backbone and the insert to renaturate the molecular clone. Plasmid C represents the final recombinant construct. The protected primers are represented by arrows (the triangles at the 5′ of the arrows stand for the nucleotide phosphorothioated bonds). Each pair of overlapping primers are represented by the same arrow type (open or closed). The restriction sites of the enzymes used in the molecular characterization of the clones are shown.

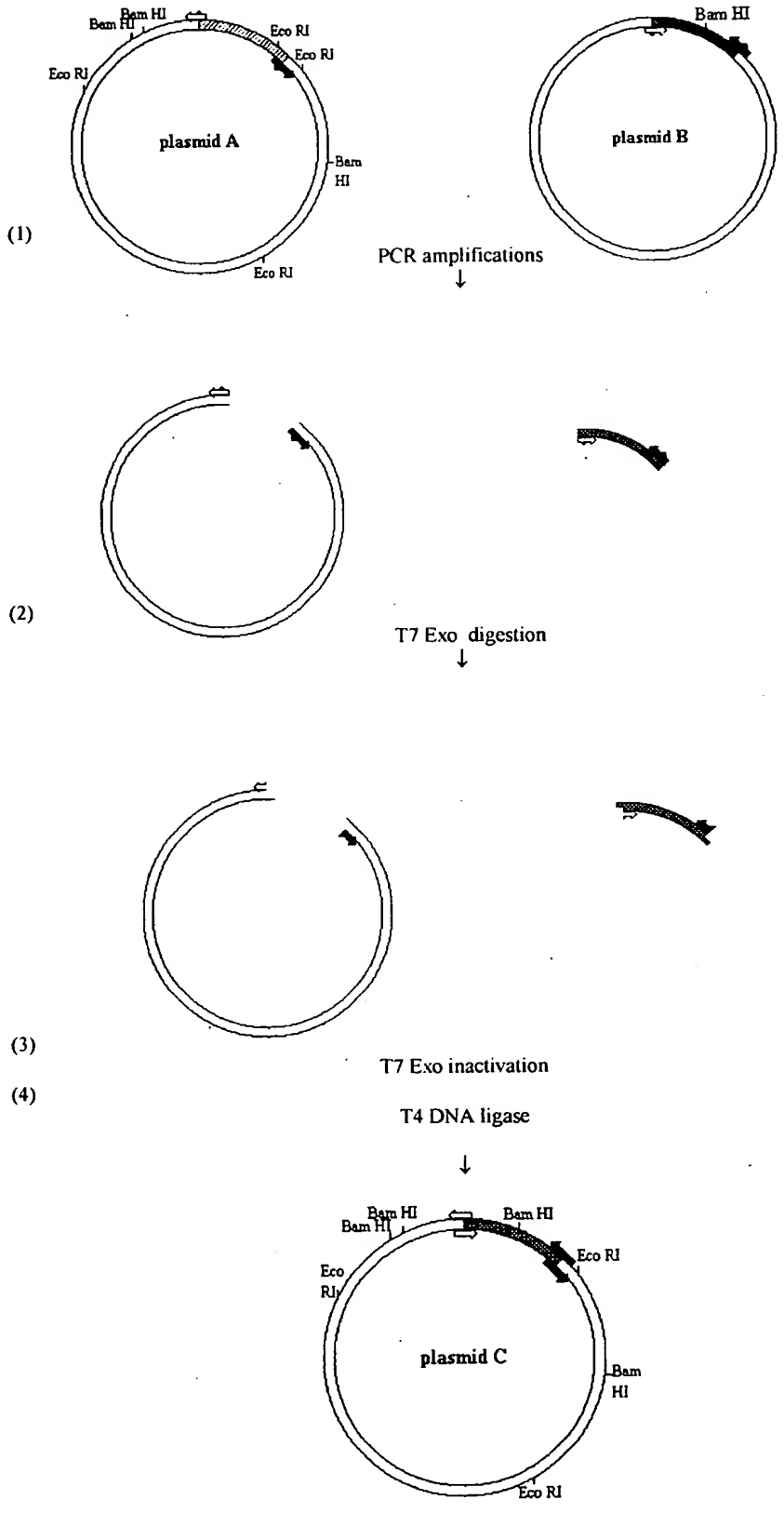

(1)
Bam HI
Bam HI
Eco RI
Eco RI
Eco RI
plasmid A
Bam HI
Eco RI
Bam HI
plasmid B
PCR amplifications
(2)
T7 Exo digestion
(3)
T7 Exo inactivation
(4)
T4 DNA ligase
Bam HI
Bam HI
Bam HI
Eco RI
Eco RI
plasmid C
Bam HI
Eco RI

Fig. 1.

tions were subjected to three cycles of 94°C for 15 s for DNA denaturation, followed by 55°C for 1 min for primer annealing and 72°C for 1 min for extension. Then 32 more cycles were carried out of denaturation (30 s at 94°C), annealing (30 s at 55°C), extension (1 min at 72°C) and finishing with 72°C for 5 min; 1/10 of each reaction volume was subjected to electrophoresis in a 0.8% agarose gel to confirm the amplifications of desired fragments (data not shown). The PCR products were purified using Quiagen Qiaquick Spin PCR Purification Kit (Quiagen, Chatsworth) following the standard protocol. For generation of single strand 3′ end of each amplified fragments, T7 Exo (US Biochemical) enzyme was added to a final concentration of 2 U mg of PCR product. The reaction tubes were incubated for 1 h at room temperature. After this incubation period the reaction tubes were placed at 70°C for 10 min in order to inactivate the enzyme. The T7 Exo digested Z6 (gp120 minus) and gp120 gene of F subtype were then combined in a single tube with a 5:1 ratio of the small to the large fragment. T4 DNA ligase was added following the kit protocol instructions (Rapid DNA Ligation Kit, Boehringer-Mannhein) and the incubation was carried out as recommended to allow the regeneration of the recombinant construct. One tenth of this ligation reaction was used to transform *E. coli* MAX efficiency DH5αF′ IQTM competent cells (Gibco-BRL, Gaithersburg), following plating on ampicillin/kanamicin-containing plates. The plasmid DNA from the colonies was isolated and the uncut plasmid DNA was fractionated by electrophoresis. All recombinants showed one band with the same electrophoretic mobility as that of the control pZ6, demonstrating that they possessed the insert (data not shown). In order to confirm the substitution, the obtained recombinant molecules were used in digestion reactions with *Eco*RI or *Bam*HI restriction enzymes and the final products fractionated on a 0.8% agarose gel (Fig. 2, lanes 1, 2 and 4, 5 respectively). As control, pZ6 was also used for digestion with these two restriction enzymes (Fig. 2, lanes 3 and 6, respectively). Since gp120 region of F subtype has a *Bam*HI restriction site that is not present on the same region of pZ6 and an *Eco*RI restriction

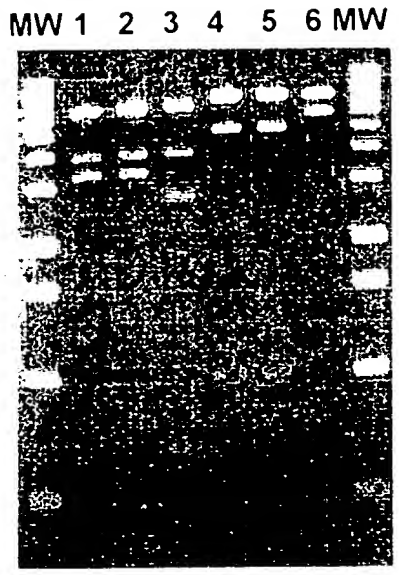


Fig. 2. Molecular characterization of the pZ6 and recombi constructs. In order to confirm the gp120 substitution at the pZ6 two clones obtained after manipulation were digested with *Eco*RI or *Bam*HI and the digestion pattern compared with that of pZ6. Lanes 1 and 2, 4 and 5: clones pZ6 gp120F1 and pZ6gp120/F2 digested with *Eco*RI and *Bam*HI respectively. Lanes 3 and 6: digestion of pZ6 with *Eco*RI and *Bam*HI, respectively. MW, molecular weight (1-kb ladder).

site present in the latter is absent in the F subty[illegible]. two different restriction patterns could be distinguished from the recombinant molecule when compared with pZ6, therefore leading to the conclusion that the recombinant constructs possess the gp120 of F subtype. In addition, the recombinants were characterized by dideoxynucleotide sequencing. Both junction boundaries between [illegible] PCR products and the entire gp120 F gene were identical to those expected for the procedure, providing evidence that the method of generating new recombinants is effective since none of the alterations as insertions, deletions or mutations were observed in this construct (data not shown).

To determine whether the recombinant pZ6 gp120 F is able to produce viral particles as it the pZ6 itself, 1 mg of each plasmid preparation was transfected into Cos-7 cells following the lipofectamine protocol (Gibco-BRL, Gaithersburg). Aliquots of cell-free supernatants were collected 24, 48, 72 and 96 h after transfection and the

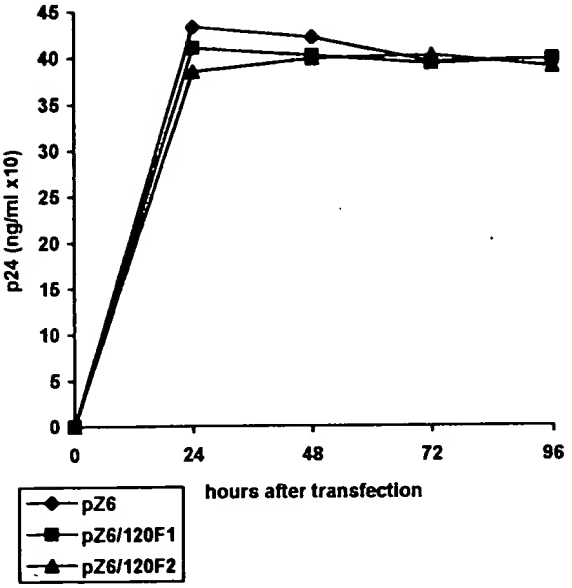


Fig. 3. Kinetics of virus production on transfected Cos-7 cells. One microgram of pZ6 (◆), pZ6/gp120F1 (■) or pZ6/gp120F2 (▲) proviral DNA was used for transfection and the virus production monitored at 1-day intervals by HIV-1 $p24^{gag}$ in cell-free supernatants.

presence of virus monitored by Coulter HIV-1 $p24^{gag}$ antigen enzyme-linked immunosorbent assay (ELISA) (Coulter Immunology). As seen in Fig. 3 the recombinant virus yield was similar to that observed for the pZ6 used as control. The virus particles released in the medium were able to establish successful infection in HIV permissive CD4 + cells (data not shown), demonstrating the viability of the construct and the success of the method.

A new procedure is described to introduce genetic changes into any recombinant DNA clone with surgical precision without the requirement of the presence of restriction sites, overcoming the drawback of the current approaches. Although another method of cloning and/or modification of any DNA sequence independent of naturally occurring restriction sites has already been described (Kerstien and Sorge, 1996), that approach uses specific primers containing a site for a type IIS enase (Eam 1104I), requiring the utilization of modified deoxynucleotides (5-methyl-deoxycytosine) during PCR reactions to protect all internal restriction sites for this enase present in the template molecules. However, this type of strategy is less efficient and time consuming, since the PCR reaction (the critical step of the method) tends to be inhibited by the presence of methylated deoxynucleotides (Wong and McClelland, 1991). The advantage of the method described here is the generation of very precise recombinants in a nucleotide sequence context close to that found in nature in a simply and efficient way requiring few manipulation steps.

Acknowledgements

We thank Dr. Mark Rayfield for helpful discussion of this manuscript, and Ivonilda Messias Santo for technical assistance.

References

Kerr, C., Sadowski, P.D., 1972. Gene 6 exonuclease of bacteriophage T7. J. Biol. Chem. 247, 311–318.

Kerstien, A.P., Sorge, J.A., 1996. Creating seamless junctions independent of restriction sites in PCR cloning. Gene 168, 31–35.

Nikiforov, T.T., Rendle, R.B., Kotewicz, M.L., Rogers, Y.-N., 1994. The use of phosphorothioated primers and exonuclease hydrolysis for the preparation of single-strand PCR products and their detection by solid-phase hybridization. Research 3, 285–291.

Srinivasan, A., Anand, R., York, D., Ranganathan, P., Feorino, P., Schochetman, G., Curran, J., Kalyanaraman, V.S., Luciw, P.A., Sanchez-Pescador, R., 1987. Molecular characterization of human immunodeficiency virus from Zaire: nucleotide sequence analysis identifies conserved and variable domains in the envelope gene. Gene 52, 71–82.

Wong, K.K., McClelland, M., 1991. PCR with 5-methyl-dCTP replacing dCTP. Nucleic Acid Res. 11, 1081–1085.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 18, Issue of September 25, pp. 11165-11173, 1983
Printed in U.S.A

Two Forms of the DNA Polymerase of Bacteriophage T7*

(Received for publication, March 21, 1983)

Michael J. Engler‡, Robert L. Lechner§, and Charles C. Richardson

From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

The DNA polymerase induced by bacteriophage T7 can be isolated in two different forms. The distinguishing properties are: 1) the specific activities of the associated 3′ to 5′ single- and double-stranded DNA exonuclease activities, 2) the ability to catalyze DNA synthesis and strand displacement at nicks, and 3) the degree of stimulation of DNA synthesis on nicked, duplex DNAs by the gene 4 protein of phage T7. Form I is obtained when purification is carried out in the absence of EDTA while Form II is obtained if all purification steps are carried out in the presence of 0.1 mM EDTA. Form I has low levels of both exonuclease activities, less than 5% of those of Form II. Form I can initiate DNA synthesis at nicks leading to strand displacement, a consequence of which is its ability to be stimulated manyfold by the helicase activity of gene 4 protein on nicked, duplex templates. On the other hand, Form II cannot initiate synthesis at nicks even in the presence of gene 4 protein. In keeping with its higher exonuclease activities, Form II of T7 DNA polymerase has higher turnover of nucleotides activity (5-fold higher than Form I) and exhibits greater fidelity of nucleotide incorporation, as indicated by the rate of incorporation of 2-aminopurine deoxynucleoside monophosphate. Both forms of T7 DNA polymerase exhibit higher fidelity of nucleotide incorporation than bacteriophage T4 DNA polymerase. In the absence of EDTA or in the presence of $FeSO_4$ or $CaCl_2$, Form II irreversibly converts to Form I. The physical difference between the two forms is not known. No difference in molecular weight can be detected between the corresponding subunits of each form of T7 DNA polymerase as measured by gel electrophoresis in the presence of sodium dodecyl sulfate.

Bacteriophage T7 provides a model for understanding the mechanisms involved in the replication of a linear, duplex DNA molecule. Within a 12-min period after infection, the 40,000-base pair T7 DNA molecule is duplicated several hundred times to give rise to a burst of 200 phage particles. To accomplish this feat, T7 has evolved an extremely efficient and economical mechanism for the replication of its DNA. The T7 genome is sufficiently large to encode its own replication proteins, bypassing the host replication machinery. Two proteins, the T7 DNA polymerase and gene 4 protein, play a major role at the replication fork and can account for many of the events known to occur *in vivo* (1).

T7 induces the synthesis of its own DNA polymerase upon infection of *Escherichia coli* (2, 3). The T7 DNA polymerase has been purified to near homogeneity (2, 4–6) and shown to be composed of two subunits (4, 7, 8). One subunit is the 84,000-Da polypeptide specified by gene 5 of the phage, while the other is the 12,000-Da thioredoxin specified by the *trxA* gene of *E. coli* (9). In addition to catalyzing the polymerization of nucleotides, T7 DNA polymerase has two associated exonuclease activities (6, 10). The enzyme possesses both single-stranded and double-stranded DNA exonuclease activities, both of which hydrolyze DNA in a 3′ to 5′ direction.

Both the T7 gene 5 protein and the *E. coli* thioredoxin have been purified to near homogeneity from cell extracts by monitoring the ability of each to restore polymerase activity to fractions containing the other subunit (7, 11). The purified subunits have also been obtained by dissociating homogeneous T7 DNA polymerase into biologically active subunits using guanidine HCl (6). Neither the gene 5 protein nor thioredoxin itself has detectable DNA polymerase activity (6, 11). Gene 5 protein alone has the 3′ to 5′ single-stranded DNA exonuclease found in T7 DNA polymerase, but not the double-stranded DNA exonuclease activity (6, 10). The addition of thioredoxin to the gene 5 protein restores the double-stranded DNA exonuclease activity as well as the polymerase activity. In fact, thioredoxin and gene 5 protein together form a molecular complex indistinguishable from T7 DNA polymerase synthesized *in vivo* (11).

The gene 4 protein of phage T7 has multiple activities. It is a single-stranded DNA-dependent nucleoside 5′-triphosphatase (12), a helicase (12), and a primase (13–18). The helicase activity of the gene 4 protein was initially inferred from its ability to stimulate T7 DNA polymerase using nicked,[1] duplex DNA as a template, a reaction that requires the hydrolysis of NTPs to NDPs and P_i (12). Subsequent studies showed that synthesis in the presence of the two proteins is initiated at nicks in the duplex DNA and that the polymerization reaction is accompanied by strand displacement (19). In the presence of rNTPs, the gene 4 protein, acting as a primase, recognizes specific pentanucleotide sequences on the displaced strand (14) and synthesizes tetranucleotide primers (13–18), which in turn are extended by T7 DNA polymerase to yield Okazaki fragments. Both the helicase and primase activities of the gene 4 protein require the hydrolysis of NTPs, a reaction that requires the presence of single-stranded DNA. The hydrolysis of NTP is, in turn,

* This investigation was supported by United States Public Health Service Grant AI-06045 and Grant NP-1L from the American Cancer Society, Inc. This is Paper 23 in a series entitled "Replication of Bacteriophage T7 Deoxyribonucleic Acid." The previous paper is Ref. 48. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of National Institutes of Health Fellowship 5 F32 AI05803. Present address, Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, TX.

§ Recipient of National Institutes of Health Fellowship 5 F32 GM07288.

[1] A nick in DNA is defined here as a single phosphodiester bond interruption in which a 5′-phosphoryl group and a 3′-hydroxyl group are present.

coupled to the 5′ to 3′ unidirecti nal translocation of the gene 4 protein along a DNA strand (1, 14).

A distinguishing feature of our initial prep:rations of T7 DNA polymerase is their ability to be stimulated by gene 4 protein to c py-nicked, duplex DNA; gene 4 pr tein is without effect n *E. coli* DNA polymerases I, II, and III, or T4 DNA polymerase (20). However, Fischer and Hinkle (21), using T7 DNA polymerase purified by a different procedure, found that the polymerase could not use nicked, duplex DNA as a primer-template even in the presence of gene 4 protein. Stratling and Knippers (22) had reported earlier that their preparation of T7 DNA polymerase could not be stimulated by gene 4 protein.

In this paper, we show that the two forms of T7 DNA polymerase exhibit several distinguishing properties. Using a new, sensitive assay we have found that Form I of T7 DNA polymerase, representative of our earlier preparations of the enzyme, catalyzes limited strand displacement synthesis at nicks in duplex templates, a reaction that is characterized in detail in the following paper (23). Form II of T7 DNA polymerase, purified using procedures in which EDTA is present in all the buffers, catalyzes little, if any, net synthesis at a nick. Form I can be stimulated by gene 4 protein at nicks, while Form II cannot. Finally, we show that Form II has 20- to 50-fold higher levels of both single- and double-stranded exonuclease activities than Form I of T7 DNA polymerase. Even with this large difference in exonuclease activities, the fidelity of both forms exceeds that of T4 DNA polymerase.

While the various distinguishing properties may seem unrelated to one another, we believe that all of the differences are the result of a single change in the enzyme. In the accompanying paper (24), we show that a displaced single strand is required for the helicase activity of gene 4 protein. Thus, the inability of Form II to be stimulated by gene 4 protein is a consequence of its inability to catalyze strand displacement synthesis at nicks. We suspect that the relatively high exonuclease activity of Form II may decrease the strand displacement activity of the enzyme (see "Discussion"). As Fischer and Hinkle (21) reported and we show here, the form of the polymerase isolated from extracts of cells infected with phage T7 is largely determined by the presence or absence of EDTA in the buffers used during purification. In fact, purified Form II may be converted to Form I by removal of EDTA during prolonged dialysis. However, no physical or chemical differences between the two forms have been found.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains and Bacteriophages—*E. coli* D110 *Su⁻ thy end polA1* has been described previously (25). *E. coli* O11′ *Su⁺ thy*, and T7 phage were obtained from Dr. F. W. Studier (Brookhaven National Laboratory). T7 amber mutants are designated by subscript notation indicating the mutant gene only. The amber mutations used are: gene 3, am29; gene 4, am20; gene 6, am147. T7 phages were grown on *E. coli* O11′ as described by Studier (26–28).

DNA—Unlabeled and ^{3}H-labeled DNA from bacteriophage T7 was prepared as previously described (29). Bacteriophage φX174am3 DNA was isolated by the method of Hutchison and Sinsheimer (30). Plasmid pBR322 DNA was prepared as previously described (31).

Nucleotides—Unlabeled nucleotides were purchased from P-L Biochemicals. 2-Aminopurine deoxynucleoside [α-^{32}P]triphosphate was a gift from Dr. M. J. Bessman (Johns Hopkins University). Other radiolabeled nucleotides were obtained from New England Nuclear.

Enzymes—Bacteriophage T7 gene 4 protein was Fraction V (50% pure) of Kolodner *et al.* (20). The specific activity of the preparation was 2500 units/mg, assayed as previously described (20). T7 DNA ligase was purified by a procedure to be published elsewhere.[2] Alkaline phosphatase from *E. coli* was obtained from Worthington and further purified (32). T4 polynucleotide kinase was fraction VI of the procedure of Richardson (33).

Other Materials—Polyethyleneimine cellulose thin layer plates were purchased from Brinkmann. Ultrapure ammonium sulfate and sucrose were from Schwarz/Mann. Streptomycin sulfate was from Merck.

Methods

DNA Polymerase Assay—T7 DNA polymerase was assayed as described by Adler and Modrich (6). One unit of polymerase activity catalyzes the incorporation of 10 nmol of total nucleotide into an acid-insoluble form in 30 min under the conditions of the assay (2).

Exonuclease Assays—Exonuclease assays were performed as described by Adler and Modrich (6). Double-stranded and single-stranded DNA exonuclease activities were measured using native T7 [^{3}H]DNA or denatured single-stranded T7 [^{3}H]DNA, respectively. One unit of exonuclease catalyzes the acid solubilization of 10 nmol of nucleotide in 30 min under the conditions of the assay.

RNA-primed DNA Synthesis—Gene 4 protein-primed DNA synthesis on single-stranded DNA templates was carried out in a reaction mixture containing T7 gene 4 protein and rNTPs in addition to T7 DNA polymerase and dNTPs. Incubation mixtures (0.05 ml) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 150 μM dCTP, dGTP, dATP, rATP, rUTP, rGTP, rCTP, and [^{3}H]dTTP (66 cpm/pmol), 20 mM KCl, 2 nmol of single-stranded φX174 DNA, 0.8 unit of T7 DNA polymerase, and gene 4 protein as indicated. After incubation at 30 °C for 30 min, acid-insoluble radioactivity was determined as previously described (34).

Stimulation of T7 DNA Polymerase by Gene 4 Protein on Nicked, Duplex Templates—Reaction mixtures (0.1 ml) contained 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 150 μM dCTP, dGTP, dATP, and [^{3}H]TTP (82 cpm/pmol), 100 μg/ml bovine serum albumin, 6 nmol of T7 DNA, 0.1 unit of T7 DNA polymerase, and gene 4 protein as indicated. After incubation at 30 °C for 20 min, acid-insoluble radioactivity was determined as previously described (34).

Turnover and Nucleotide Selectivity Assays—Turnover is defined as the DNA-dependent conversion of deoxynucleoside triphosphates into their corresponding monophosphates. Reaction volumes were adjusted for the number of time points necessary for the experiment. Each incubation mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 300 μM nucleoside triphosphates as indicated, 4 units/ml of either Form I or Form II of T7 DNA polymerase, and the indicated DNA template. The concentration of denatured salmon sperm DNA was 200 μg/ml. The concentration of the duplex circular DNA bearing a preformed, topologically stable replication fork (23) was 3 μg/ml. Incubation temperature was 37 °C.

Nucleotide concentrations in the turnover assay were determined by thin layer chromatography (35). Aliquots (10–15 μl) were removed, added to 5 μl of a solution containing 50 mM EDTA and dATP and dAMP as markers, applied to a polyethyleneimine plate, and developed with 1 M LiCl. Ultraviolet light-absorbing spots were cut out and radioactivity was determined by liquid scintillation counting.

Purification of Enzymes—Form II of T7 DNA polymerase was purified as follows. *E. coli* D110 was grown and infected with $T7_{as6}$ as previously described[3] (36). Cell paste was suspended in 0.05 M Tris-HCl (pH 7.5), 10% sucrose to a final volume of 4 ml/g of cells. Aliquots were frozen in liquid nitrogen and stored at −85 °C.

Frozen cells (35 ml) were thawed overnight on ice and divided into two aliquots of Spinco Type 30 rotor tubes, and 0.33 ml each of 5 M NaCl and a solution of 10 mg/ml lysozyme, 50 mM Tris-HCl (pH 7.5), 10% sucrose were added to each tube. After 45 min at 0 °C, the tubes were transferred to a 37 °C water bath, heated to 20 °C with stirring, and then chilled to 5 °C in an ice bath with stirring. The lysates were then centrifuged at 30,000 rpm for 45 min in a Spinco Type 30 rotor. The A_{260} of the supernatant fluid was 140.

To 25 ml of the supernatant fluid were added 2.8 ml of freshly prepared 30% streptomycin sulfate over a 30-min period with stirring. After stirring an additional 30 min, the precipitate was removed by centrifugation.

Ammonium sulfate was then added to 50% saturation with stirring over a 30-min period. After stirring for an additional 30 min, the precipitate was collected and dissolved in 15 ml of 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% (v/v) glycerol (Buffer A) (Fraction III).

A column of Whatman DE52 DEAE-cellulose (1.8 cm² × 12 cm) was prepared and equilibrated with Buffer A containing 0.1 M NaCl.

[2] M. J. Engler, R. L. Lechner, and C. C. Richardson, unpublished results.

Fraction III (3700 units of activity) was adjusted to a conductivity less than that of the column buffer by the addition of 15 ml of Buffer A and applied to the column at a rate of 15 ml/h. The column was washed with 30 ml of Buffer A containing 0.1 M NaCl, and proteins were eluted with a 200-ml linear gradient from 0.1 to 0.5 M NaCl in Buffer A. A flow rate of 15 ml/h was maintained and fractions of 3.5 ml were collected and assayed for DNA polymerase activity. Fractions containing 80% of the total eluted activity were combined and dialyzed against 20 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM dithiothreitol, 20% (v/v) glycerol (Buffer B). The pool contained 20.2 ml and was dialyzed against 1 liter of Buffer B twice, each time for 3 h (Fraction IV).

A column of Whatman P-11 phosphocellulose (0.7 cm^2 × 7 cm) was prepared and equilibrated with Buffer B. 17 ml of Fraction IV containing 1600 units of activity were applied to the column at a rate of 4 ml/h and washed with Buffer B containing 0.1 M KCl. Proteins were eluted with a 50-ml linear gradient from 0.1 to 0.5 M KCl in Buffer B. Fractions containing 80% of the activity were pooled and concentrated by dialysis against dry Sephadex G-100 and then against Buffer B containing 50% (v/v) glycerol. The total concentration was approximately 5-fold and the last dialysis was overnight (Fraction V). Fraction V contained 870 units of polymerase activity and was stored at −20 °C.

Form II of T7 DNA polymerase has also been purified by modifying the procedure of Adler and Modrich (6). EDTA (0.1 mM) was included in all buffers starting with redissolving the ammonium sulfate precipitate (Fraction III). The only exception was that the hydroxylapatite column was equilibrated in the absence of EDTA but the sample contained EDTA and proteins were eluted using a gradient containing EDTA. A similar modification has been used by Fischer and Hinkle (21). We have detected no differences in the properties of the enzyme purified by either of these methods. Unless indicated otherwise, Form II of the T7 DNA polymerase was purified as described in detail here.

The procedure of Adler and Modrich (6) has been used to prepare Form I of the T7 DNA polymerase. This procedure, which does not include EDTA until the final concentration and storage, yields a variable mixture of the two forms of T7 DNA polymerase. We have determined the proportion of Form II by examining the level of either single-stranded or double-stranded exonuclease (see "Results") and have chosen a preparation of T7 DNA polymerase with a high fraction of Form I. Unless noted otherwise, Form I of T7 DNA polymerase was this preparation.

Form I can also be prepared from Form II by dialysis against a buffer solution containing no EDTA. We have successfully prepared Form I from Form II at the stage of the phosphocellulose pool, just prior to concentration and dialysis against the storage buffer. Portions of the phosphocellulose pool of Form II of T7 DNA polymerase (unconcentrated Fraction V) were dialyzed against 500 volumes of 20 mM potassium phosphate buffer (pH 7.4), 0.5 mM dithiothreitol, 20% glycerol at 0 °C. Dialysis for 4 days with changes of dialysis every 24 h led to greater than 90% conversion of Form II to Form I as measured by the levels of exonuclease activity (see "Results").

Preparation of Nicked, Circular DNA Containing 5′-^{32}P-Phosphomonoesters—Plasmid pBR322 DNA was nicked with the restriction enzyme *Eco*RI as described in the following paper (23), and labeled by the method of Weiss *et al.* (32), First, phosphomonoesters were hydrolyzed by incubation with bacterial alkaline phosphatase. The incubation mixture (1.0 ml) contained 50 μg of *Eco*RI-nicked pBR322 DNA, 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, and 1 unit of alkaline phosphatase. The mixture was incubated for 30 min at 65 °C with additional phosphatase (1 unit) added at 10 and 20 min of incubation. Next, 5′-^{32}P-phosphomonoesters were introduced by incubation with polynucleotide kinase and [γ-^{32}P]ATP. The reaction mixture (1.25 ml) contained 50 μg of phosphatase-treated DNA, 28 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 17 mM 2-mercaptoethanol, 1.4 mM potassium phosphate buffer (pH 7.5), 17 μM [γ-^{32}P]ATP (6 × 10^{10} cpm/μmol), and 75 units of T4 polynucleotide kinase. The mixture was incubated for 45 min at 37 °C with additional polynucleotide kinase (30 units) added at 15 and 30 min. The DNA was extracted once with phenol and then with chloroform/isoamyl alcohol (24:1). Unreacted [γ-^{32}P]ATP was removed by gel filtration using a Sephadex G-75 column.

Other Methods—Protein was determined by the procedure of Lowry *et al.* (37) using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed according to Laemmli (38) using the slab gel system of O'Farrell (39). The resolving gel consisted of two parts. The bottom half was 17% polyacrylamide and the top half was 10% polyacrylamide. The stacking gel was 4.75% polyacrylamide. Gels were stained with Coomassie brilliant blue as described by Fairbanks *et al.* (40). DNA concentrations were determined from reported specific absorbances at 260 nm and are expressed as nucleotide equivalents. Denatured T7 DNA was prepared by heating T7 DNA in a boiling water bath and quickly cooling to 0 °C. All pH measurements were made at room temperature at a buffer concentration of 0.05 M.

RESULTS

Purification and Assay of Form I and Form II of T7 DNA Polymerase—T7 DNA polymerase has been purified by a variety of procedures by monitoring the activity of the polymerase on denatured DNA templates (2–6, 21). Only the study of Fischer and Hinkle (21) recognized the existence of an alternative form of the polymerase that could not be stimulated by gene 4 protein on nicked, duplex DNA templates. The purification procedure devised here for Form II of T7 DNA polymerase arose as a result of our attempts to purify a factor that would prevent strand displacement or a form of T7 DNA polymerase that would not carry out strand displacement at a nick. Such a reaction, catalyzed by Form I and described in the following paper (23), frustrated our early attempts to process and join Okazaki fragments (41). Concurrent studies on the mechanism of action of the helicase activity of the gene 4 protein of phage T7 suggested to us that the inability of the polymerase purified by Fischer and Hinkle (21) to be stimulated by gene 4 protein at nicks was, in fact, a consequence of its inability to carry out strand displacement synthesis. As is shown in the accompanying paper (23), we feel that Form II of T7 DNA polymerase described here is the same as the DNA polymerase described by Fischer and Hinkle (21). Similar to their procedure, we had included EDTA in our column buffers and, as will be shown below, this is the significant difference in the purification procedure for Form II of T7 DNA polymerase. The procedure devised by Adler and Modrich (6) will also yield Form II provided 0.1 mM EDTA is included in the buffers during purification.

Form I can be prepared directly from Form II by prolonged dialysis in the absence of EDTA as described below. However, most preparations of T7 DNA polymerase purified in the absence of EDTA yield either a mixture of Form I and Form II or almost exclusively Form I. Here we have used the procedure of Adler and Modrich (6) to obtain a preparation of T7 DNA polymerase which, as judged by several criteria described below, is almost exclusively Form I. We emphasize, however, that other preparations of T7 DNA polymerase purified by the same procedure have contained variable amounts of Form I, presumably due to the slow conversion to Form I in the absence of EDTA.

A simple assay for the presence of Form I is the measurement of the stimulation of T7 DNA polymerase at nicks by gene 4 protein; gene 4 protein has no effect on DNA synthesis catalyzed by Form II on such a primer-template. Measurement of either the single- or double-stranded DNA exonuclease activities of the polymerase provides a quantitative measurement of Form II since Form I has less than 5% of the exonuclease activity of Form II.

Purity and Subunit Composition—The most purified preparations of Form I and Form II represent a 470- and 1,000-fold purification, respectively, over the crude extract. The specific activities of the DNA polymerase activities of Forms I and II on denatured salmon sperm DNA in the standard assay are 5,800 and 10,300 units/mg of protein, respectively.

We have subjected purified preparations of both Form I and Form II to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The subunit stoichiometry for both forms of DNA polymerase was approximately 1.0 mol of thioredoxin/mol of T7 gene 5 protein as estimated by

scanning microdensitometry of Coomassie blue-stained gels (Fig. 1, *A* and *B*). This value is in agreement with the values previously reported (4, 6). When Form I and Form II were analyzed together on the same gel, no difference in the mobility of either gene 5 protein or thioredoxin could be detected (data not shown). We estimate that this method could have resolved a 1500-Da difference.

Form I has been stored in 50% glycerol at −18 °C for up to 2 years without appreciable loss of activity (<10%). Form II has been similarly stored for 1 year without loss of DNA polymerase activity (<10%) and without any detectable conversion to Form I (<10% as measured by stimulation by T7 gene 4 protein).

Effect of Helicase Activity of T7 Gene 4 Protein on DNA Synthesis Catalyzed by Forms I and II—Helicase activity of the gene 4 protein was initially inferred from its ability to stimulate T7 DNA polymerase on nicked, duplex DNA templates (19). Such an assay provides a sensitive means of distinguishing Form I from Form II. As shown in Fig. 2, DNA synthesis is limited with either form of T7 DNA polymerase

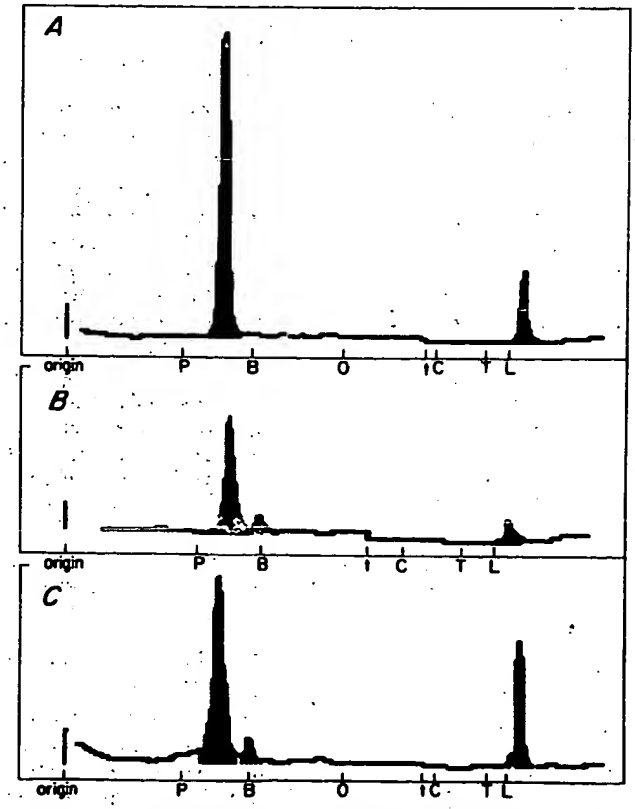


FIG. 1. **Microdensitometer tracings of sodium dodecyl sulfate-polyacrylamide gel electrophoretic banding patterns of Form I and Form II of T7 DNA polymerase.** Protein samples were heated for 2 min in a boiling water bath in a solution containing 2.5% sodium dodecyl sulfate and 3% 2-mercaptoethanol, and then subjected to electrophoresis as described under "Experimental Procedures." Each electrophoretic run included a parallel lane of protein markers, the positions of which are denoted on the *abscissa*: *P*, phosphorylase *b*; *B*, bovine serum albumin; *O*, ovalbumin; *C*, carbonic anhydrase; *T*, trypsin inhibitor; and *L*, lysozyme. *t* marks the transition from 10 to 17% polyacrylamide concentration. *A*, purified Form II of T7 DNA polymerase. *B*, Form I of T7 DNA polymerase obtained by dialysis of Form II for 1 week at 0 °C against a buffered solution minus EDTA. *C*, Form I of T7 DNA polymerase obtained by dialysis of Form II of T7 DNA polymerase for 3 weeks against a buffered solution minus EDTA as described under "Experimental Procedures." This sample was concentrated 3-fold by vacuum evaporation prior to electrophoresis.

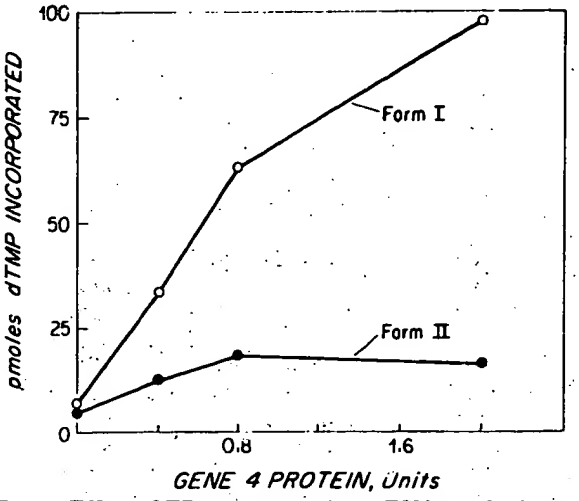


FIG. 2. **Effect of T7 gene 4 protein on DNA synthesis catalyzed by Forms I and II on nicked, duplex DNA templates.** Native T7 DNA was incubated with either Form I (○) or Form II (●) of T7 DNA polymerase, dNTPs, and the indicated amounts of gene 4 protein. Incubations were performed as described under "Experimental Procedures."

using nicked, duplex DNA as template. However, the addition of purified gene 4 protein to a reaction mixture containing Form I of T7 DNA polymerase markedly stimulates DNA synthesis. This result is identical with that previously described for T7 DNA polymerase (19), indicating that our previous preparations of T7 DNA polymerase were predominantly Form I. By contrast, gene 4 protein is virtually without effect on Form II (Fig. 2).

Form I of T7 DNA Polymerase Performs Strand Displacement Synthesis—In earlier studies on T7 DNA polymerase, we concluded from an incorporation assay that T7 DNA polymerase could not initiate DNA synthesis at nicks in duplex DNA templates (19). A more sensitive assay for DNA synthesis indicates that this is not the case for Form I of T7 DNA polymerase. Alteration of the nick by strand displacement synthesis from the 3′-hydroxyl group will prevent DNA ligase from incorporating the 5′-phosphoryl group into a phosphodiester linkage. We have detected this alteration by using ^{32}P-labeled phosphomonoesters at nicks in an assay in which bacterial alkaline phosphatase is used to release phosphomonoesters that are not converted into phosphodiesters by DNA ligase. As shown in Fig. 3, incubation of such a substrate with Form I of T7 DNA polymerase, under conditions of DNA synthesis, destroys its ability to act as a substrate for T7 DNA ligase. Incubation with Form II, on the other hand, did not affect the susceptibility of the DNA substrate to ligase. Furthermore, incubation with DNA polymerase without subsequent treatment with phosphatase did not affect the amount of acid-precipitable radioactivity, thus eliminating the possibility of nucleolytic degradation of the substrate.

In an independent experiment (data not shown), nicks introduced by *Eco*RI (5′-G/AATTC-3′) were protected from destruction by Form I when incubation was carried out in the presence of only three deoxynucleoside 5′-triphosphates, dCTP, dTTP, and dGTP. In the presence of dGTP, dTTP, and dATP, however, the nicks were destroyed as measured by their ability to be eliminated by DNA ligase. We conclude that DNA synthesis is catalyzed by Form I at a nick and that the newly synthesized DNA displaces the 5′ terminus.

In the accompanying paper (23), we show that Form I of T7 DNA polymerase catalyzes limited synthesis of a few

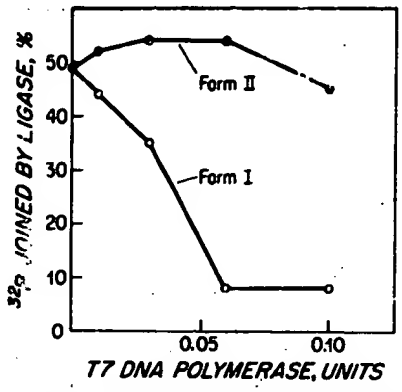


FIG. 3. **Incubation with Form I causes the loss of ligase-susceptible nicks.** Duplex DNA containing nicks bearing ^{32}P-labeled 5′-phosphomonoester was incubated for 15 min at 30 °C with various amounts of either Form I (○) or II (●) of T7 DNA polymerase. The reaction mixtures (0.1 ml) contained 40 mM Tris-HCl (pH 7.5), mM $MgCl_2$, 10 mM 2-mercaptoethanol, 150 μM dATP, TTP, dGTP, dCTP, and ATP. T7 DNA ligase (0.09 pyrophosphate exchange unit (31)) was added to all reactions and incubation continued for 30 min. The amount of phosphodiester linkage formation (per cent joining) by T7 DNA ligase was determined by measuring the formation of phosphatase-resistant ^{32}P as described by Weiss *et al.* (32).

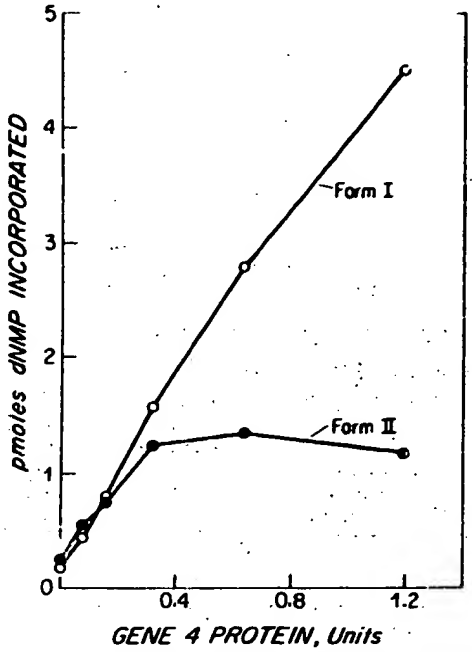


FIG. 4. **RNA-primed DNA synthesis on single-stranded circular DNA.** Either Form I (○) or Form II (●) of T7 DNA polymerase was incubated with φX174 DNA, all four dNTPs and rNTPs, and the indicated amounts of gene 4 protein. Incubations were performed as described under "Experimental Procedures."

hundred nucleotides from a nick resulting in strand displacement, eliminating the nick and creating a 5′-single-stranded tail. It is, in fact, this single-stranded tail that allows gene 4 protein to serve as a helicase and stimulate Form I. Given such a preformed tail, gene 4 protein stimulates DNA synthesis catalyzed by Form II equally well (24).

RNA-primed DNA Synthesis on Single-stranded Circular DNA—The gene 4 protein of bacteriophage T7 recognizes specific sequences on single-stranded DNA and then catalyzes the synthesis of tetraribonucleotide primers complementary to the template (14). T7 DNA polymerase, like all DNA polymerases, is unable to initiate DNA synthesis *de novo*. However, it readily uses the RNA primers synthesized by the gene 4 protein to begin synthesis (13, 15, 17, 18). In order to determine if both Forms I and II of T7 DNA polymerase can use the tetraribonucleotide primers synthesized by gene 4 protein, we examined RNA-primed DNA synthesis on the single-stranded, circular DNA of phage φX174. On this template molecule, lacking a 3′-hydroxyl terminus, there is an absolute requirement for RNA primers synthesized by the gene 4 protein for DNA synthesis (14).

As shown in Fig. 4, DNA synthesis by both forms of T7 DNA polymerase requires primer synthesis by the gene 4 protein. Furthermore, with limited primer synthesis at low concentrations of gene 4 protein, the primers are used equally well by both forms of T7 DNA polymerase. There is, however, a striking difference in the response of the two forms to increased amounts of gene 4 protein. Form I is increasingly stimulated by the addition of gene 4 protein, and the amount of DNA synthesis exceeds twice the amount of template present (Fig. 4). With Form II, on the other hand, DNA synthesis stops at a level that approximates one round of DNA synthesis. The extensive synthesis obtained with Form I is a consequence of the ability of this form to perform strand displacement synthesis, leading to a rolling circle mode of replication (19).

Exonuclease Activities of the Two Forms—Purified preparations of T7 DNA polymerase, either isolated in the native form (6) or reconstituted from the individual subunits (10), have a 3′ to 5′ exonucleolytic activity on both single-stranded and duplex DNA. The purified gene 5 protein has only the single-stranded DNA exonuclease activity, while the reconstituted polymerase has both activities. Two lines of evidence support the identity of the exonuclease activities with the polymerase: 1) both exonuclease activities chromatograph precisely with DNA polymerase activity during purification and 2) the exonuclease activities are inhibited by dNTPs (6, 10).

Form I and Form II of T7 DNA polymerase can be distinguished by their levels of exonuclease activity. Both the single- and double-stranded DNA exonuclease-specific activities of Form I are less than 5% of those observed with Form II (Fig. 5). The specific activities of single-stranded DNA exonuclease activities for Forms I and II are 12 and 328 units/mg, respectively; the specific activities of the double-stranded DNA exonuclease activities for Forms I and II are 20 and 918 units/mg, respectively.

Nucleotide Turnover—In view of the difference in exonuclease levels of the two forms of T7 DNA polymerase, we have studied the rate of "nucleotide turnover" and selectivity of both forms to determine if the specific activity of the exonuclease affects the turnover and fidelity as it does for T4 DNA polymerase. Nucleotide turnover is the DNA-dependent conversion of a deoxynucleoside triphosphate to the corresponding deoxynucleoside monophosphate. Such a measurement is made under conditions of DNA synthesis, and thus reflects the levels of "editing" (35, 42).

In the experiment shown in Table I, the incorporation of radioactively labeled dAMP moieties into DNA and the formation of dAMP were measured at 30 °C. The turnover of dAMP is also expressed as the percentage of total nucleotide inserted by the polymerase that was subsequently removed. With denatured salmon sperm DNA as primer-template, Form II exhibits a much greater turnover than does Form I, as one would have predicted from its level of exonuclease activity (see Fig. 5). So great is the turnover by Form II on this template that 7 of every 10 nucleotides that are inserted by the enzyme are removed. Form I, on the other hand,

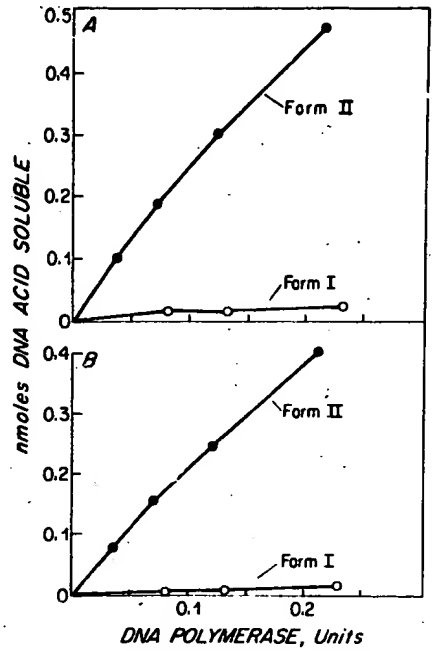


FIG. 5. **Exonuclease activities of the two forms of T7 DNA polymerase on single-stranded and duplex DNA.** Exonuclease assays were performed as described under "Experimental Procedures" with both single-stranded (*A*) and duplex DNA (*B*) using Form I (○) or Form II (●) of T7 DNA polymerase.

TABLE I

Incorporation and turnover of dAMP by the two forms of T7 DNA polymerase

Per cent turnover = 100 × turnover/(turnover + incorporation). Incorporation of nucleotides and nucleotide turnover were measured as described under "Experimental Procedures." The preformed, topologically stable replication fork (24) is described in the text. Incubations were for 10 min at 30 °C. Aliquots (10 μl) were used for both incorporation and turnover determinations. The specific activity of the [^{3}H]dATP was 75 cpm/pmol.

Primer-template and additions	Form I			Form II		
	Incorporation	Turnover	Turnover	Incorporation	Turnover	Turnover
	pmol	*pmol*	%	*pmol*	*pmol*	%
Single-stranded DNA	18.4	0.8	4	28.8	66.3	70
Preformed replication fork and gene 4 protein	13.0	0.5	4	31.3	8.8	22

removes only 4 of every 100 nucleotides it inserts, reflecting its much lower exonuclease activity.

Since T7 DNA polymerase is unusual in that it possesses a double-stranded DNA-specific exonuclease, it seems possible that idling at an end or before a duplex region in the DNA could result in a large amount of nucleotide turnover which would not reflect editing activity. To decrease the contribution of idling to dAMP turnover, we have measured turnover using a model replication fork that contains no ends or nicks (see the accompanying paper (24) for a description of a topologically stable replication fork). This synthetic replication fork was reacted with each form of T7 DNA polymerase in the presence of T7 gene 4 protein. As shown in Table I, the turnover of dAMP for Form I remained unchanged from the value obtained when salmon sperm DNA was used as primer-template (4%), while the turnover of Form II decreased 3-fold to a value of 22%. Since this reaction more closely mimics leading strand synthesis at a replication fork, it is possible that this turnover measurement is indicative of editing activity.

Fidelity—The misincorporation of the base analog 2-aminopurine in competition with adenine has been used as an *in vitro* test of the fidelity of DNA synthesis (43). 2-Aminopurine is a close analog of adenine and is incorporated at a measurable frequency, yet it is not incorporated as readily as adenine by any tested DNA polymerase. The amount of 3′ to 5′ exonuclease associated with T4 DNA polymerase purified from wild type phages and phages with mutations in the structural gene for the DNA polymerase has been correlated with the *in vivo* mutation rates observed in these phages (43). Using these methods, it has been shown that the 3′ to 5′ exonuclease activity plays a role in replication fidelity by T4 DNA polymerase. We have therefore compared the *in vitro* accuracy of the two forms of T7 DNA polymerase with that of T4 DNA polymerase.

The results of the fidelity test of Forms I and II of T7 DNA polymerase and of T4 DNA polymerase are shown in Table II. The replication discrimination, and hence fidelity, is invariant with the concentration of dATP and 2-aminopurine deoxynucleoside triphosphate between 50 and 400 μM as long as the two nucleotides are present in equal concentration. Both forms of T7 DNA polymerase exhibit higher fidelity than T4 DNA polymerase. Form II of T7 DNA polymerase incorporates 36 correct nucleotides (dAMP) for every one incorrect nucleotide (2-aminopurine deoxynucleoside monophosphate). Form I, on the other hand, has less ability to discriminate between adenine and 2-aminopurine, but shows a high degree of fidelity, nevertheless (22:1), as compared to T4 DNA polymerase (14:1). These results are consistent with the turnover values obtained for T7 DNA polymerase Form I and Form II on a replication fork in the presence of T7 gene 4 protein. It is interesting, however, that T4 DNA polymerase, which turns over dNMP to approximately the same or greater degree as Form I of T7 polymerase, has a lower discrimination index. It has not been possible to determine the effect of gene 4 protein on fidelity since it is inhibited by 2-aminopurine deoxynucleoside triphosphate.[2]

Conversion of Form II to Form I—As first observed by Fischer and Hinkle (21), it is sufficient to add EDTA to the purification buffers to obtain Form II, free of Form I. In fact, removal of EDTA from purified Form II by prolonged dialysis results in its slow conversion to Form I. We have monitored this conversion by measuring three activities: DNA polymerase activity on single-stranded DNA, DNA polymerase activity in the presence of T7 gene 4 protein using a nicked, duplex DNA as primer-template, and DNA exonuclease activities on ^{3}H-labeled single- and double-stranded DNA substrates. As shown in Table III, dialysis of Form II (Preparation A) for two days in the absence of EDTA leads to a 6-fold increase in its activity on duplex DNA in the presence of gene 4 protein. Dialysis has no effect on polymerase activity on

TABLE II

Ratio of nucleotide incorporation

Incubations were performed as described under "Experimental Procedures." Denatured salmon sperm DNA was used as primer-template, dGTP, dCTP, and dTTP were present at 300 μM, and [^{3}H]dATP (75 cpm/pmol) and 2-aminopurine deoxynucleoside [α-^{32}P]triphosphate (125 cpm/pmol) were present at 200 μM. Incorporation of labeled nucleotides (dAMP and 2-aminopurine deoxynucleoside triphosphate) was determined as described under "Experimental Procedures." Data shown are for 20-μl aliquots taken at 15 min.

dAMP/dNTP incorporated into DNA polymerase		
T7 Form I	T7 Form II	T4 wild type
22	36	14

TABLE III

Conversion of Form II of T7 DNA polymerase to Form I

DNA polymerase activity and both single-stranded and double-stranded DNA exonuclease activities were determined as described under "Experimental Procedures." DNA synthesis originating at nicks in duplex T7 DNA was measured in a reaction mixture containing 2.0 units of T7 gene 4 protein as described under "Experimental Procedures." The number of picomoles synthesized in the absence of gene 4 protein (<6 pmol) has been subtracted. The ratio of single- or double-stranded DNA exonuclease activities to DNA polymerase activity was determined by dividing the number of units of each exonuclease activity by the number of units of each preparation of T7 DNA polymerase. Form II of T7 DNA polymerase was dialyzed for 48 h at 0 °C in the absence of EDTA as described under "Experimental Procedures." Two prepaations (A and B) of Form II were converted in this experiment. A was >95% pure, and B was 82% pure by sodium dodecyl sulfate gel electrophoretic analysis. Recovery of DNA polymerase activity from the dialysis bag was 60% after 4 days of dialysis.

Treatment	Gene 4 protein-dependent DNA synthesis from nicks	Single-stranded exonuclease/polymerase		Double-stranded exonuclease/polymerase	
		A	B	A	B
	pmol				
None	12	0.053	0.071	0.16	0.20
2 days	112	—[a]	—	0.049	0.063
4 days	—	0.005	0.008	0.015	0.020

[a] —, not determined.

single-stranded DNA templates. Concomitant with this stimulation by gene 4 protein, there is a loss of both single- and double-stranded exonuclease activities; after 4 days of dialysis, the ratio of these nuclease activities to DNA polymerase activity decreases 10-fold, indicating roughly a 90% conversion of Form II to Form I. This result may indicate that a single change in the enzyme is responsible for all the measurable differences. Preparations A and B, although differing in purity, converted at nearly identical rates.

As discussed earlier, we have not been able to resolve a molecular mass difference between the T7 gene 5 protein (84,000 Da) or the thioredoxin (12,000 Da) subunits of the two forms of the polymerase. However, we have detected polypeptides that are probably derived from the gene 5 protein during the course of dialysis. As shown in Fig. 1, dialysis of Form II (*A*) in the absence of EDTA leads to the appearance of a polypeptide having $M_r \sim 72{,}000$ (*B*) and representing approximately 7% of the total protein. Storage of this preparation of Form I at 0 °C for 2 weeks resulted in the presence of an additional polypeptide of $M_r = 13{,}000$ (*C*) and the ratio of thioredoxin to gene 5 protein increased 2-fold, suggesting a loss of gene 5 protein.

An alternative method for the conversion of Form II into Form I is the addition of particular metal salts. In one experiment, the concentration of EDTA was reduced to 0.02 mM and the concentration of metal ion was 0.05 mM. Among the salts tested, the most effective were $FeSO_4$ and $CaCl_2$. The metal salts $ZnSO_4$ and $FeCl_3$ were less effective and $CuCl_2$ had no effect on the conversion. The rate of conversion by metal ions was similar to that observed in the dialysis experiment above.

Attempts to convert Form I to Form II have been unsuccessful. For example, a similar dialysis of Form I against a buffered solution containing EDTA had no effect on the activities of the DNA polymerase.

DISCUSSION

The T7-induced DNA polymerase, initially thought to be a phage-encoded enzyme solely for the polymerization of nucleotides, has proven to be an intriguing protein. Subsequent studies have shown that the enzyme is actually composed of two subunits, one being the 84,000-Da polypeptide coded by gene 5 of the phage and the other being the 12,000-Da thioredoxin coded by the *trxA* gene of *E. coli* (4, 7–9). The physiological significance of this acquisition of a host protein is not known. In this paper, we have described an additional feature of T7 DNA polymerase; the enzyme can be isolated in two forms that we have thus far been able to distinguish only by their enzymatic properties. However, the identification of these two forms has resolved a number of puzzling observations but raised several questions regarding the biological role of the two forms.

We have observed three distinguishing features of the two forms of T7 DNA polymerases: (i) the level of associated single- and double-stranded DNA exonuclease activities; (ii) the ability to perform a limited amount of strand displacement synthesis; and (iii) the ability to be stimulated by the helicase activity of T7 gene 4 protein on nicked, duplex DNA templates. Form I has low specific activities of both exonuclease activities, it catalyzes the limited polymerization of nucleotides at nicks, and it is stimulated manyfold by gene 4 protein on nicked DNA templates. Form II, by contrast, has 20- to 40-fold greater exonuclease activity on both single- and double-stranded DNA and is unable to polymerize even one nucleotide from a 3′-hydroxyl group at a nick, and this latter reaction is unaffected by the presence of gene 4 protein.

Are all of the three seemingly unrelated properties a result of a single change in the polymerase? Although we have not yet identified physical differences in the two forms, we believe that all three properties are a consequence of a single alteration in the enzyme. In the following paper (23), we present a detailed characterization of the strand displacement reaction catalyzed by Form I and suggest that it results from the low level of exonuclease activity of this form of the enzyme. In the third paper (24) of this series, we show that the ability of Form I to be stimulated by gene 4 protein to copy duplex DNA starting at a nick is a direct consequence of the capacity of Form I to generate a displaced strand at the nick; gene 4 protein can then use this single strand to translocate to the fork where it serves as a helicase. Provided with a preformed replication fork, Form II of T7 DNA polymerase is stimulated by the gene 4 protein even more than Form I on duplex DNA (24).

Turnover, the DNA-dependent conversion of dNTP into the corresponding deoxynucleoside monophosphates, has been correlated with the 3′ to 5′ exonuclease activity of prokaryotic DNA polymerases and is believed to indicate the level of the editing exonuclease activities of these enzymes (35, 42–44). The turnover is high (70%) for Form II on denatured salmon sperm DNA. A heightened turnover rate is postulated to be the biochemical defect in the temperature sensitivity of the DNA polymerase induced by T4 L141 (45). However, we do not believe that Form II of T7 DNA polymerase is defective, since turnover is reduced to 22% if the polymerase is given a branched primer-template and is assisted by the helicase activity of the T7 gene 4 protein. Gillin and Nossal (46) have proposed that the T4 DNA polymerase isolated from cells infected with the T4 mutant CB120 is deficient in strand displacement synthesis. Their results indicate that the greater the potential secondary structure in the template, the greater the turnover of nucleotides. It has been suggested that the CB120 mutant T4 DNA polymerase repeatedly removes and reinserts nucleotides where a block is encountered. It is possible that the higher turnover observed for T7 DNA polymerase Form II reflects a similar phenomenon, but a more interesting possibility is that the double-stranded exonuclease of T7 DNA polymerase is specifically

designed to prevent strand displacement at a nick or a branch point. This possibility is suggested by the observation presented in an accompanying paper (24) that T7 DNA polymerase Form II differs strikingly from T4 DNA polymerase in strand displacement activity; although T4 DNA polymerase will catalyze strand displacement at a branch point, T7 DNA polymerase Form II catalyzes little or no net synthesis under the same conditions.

The higher turnover of dAMP observed for Form II of T7 DNA polymerase as compared to Form I could result in its higher fidelity as indicated by its rate of incorporation of 2-aminopurine deoxynucleoside monophosphate. It is of interest, however, that the fidelity of both forms of T7 DNA polymerase is greater than the fidelity of T4 DNA polymerase. Since Form I has a turnover for dATP which is equal to or less than that of T4 DNA polymerase, it is possible that selectivity of base insertion is higher for the T7 DNA polymerase than for T4 DNA polymerase, although our data could not be used to calculate an accurate insertion ratio (43).

Form II of T7 DNA polymerase has about the same level of 3′ to 5′ single strand-specific exonuclease activity as T4 DNA polymerase. Form I, on the other hand, has a much lower level of 3′ to 5′ single strand-specific exonuclease activity even though its nucleotide turnover rate is about the same. Thus, it might be coincidental that the levels of 3′ to 5′ exonuclease activity of the various "mutator" and "antimutator" strains of T4 DNA polymerase are correlated with their fidelities. In other words, the possession of a highly active single strand-specific 3′ to 5′ exonuclease activity (*i.e.* the ability to rapidly degrade single-stranded DNA as measured by the usual acid precipitation assays) is not necessarily a prerequisite for a DNA polymerase with good fidelity (44). Exonuclease activities may be required for other purposes, for example, to reestablish the DNA structure at a replication fork after it has been rearranged by branch migration.

At present, we know of two conditions that affect the conversion of Form II to Form I: the presence of EDTA and metal ions. The presence of EDTA during purification leads to a preparation that is almost exclusively Form II. Form II can be isolated in the absence of EDTA, but it is usually contaminated with variable amounts of Form I. Preparations that appear to be predominantly Form I, such as those used in this study, were often obtained using procedures that had prolonged delays between chromatographic steps. We have also found that prolonged dialysis at 0 °C to remove EDTA from Form II results in its conversion to Form I. The conversion is irreversible since the addition of EDTA to Form I is without effect. Form II will also convert to Form I in the presence of a low concentration of EDTA when metal salts are added in excess. However, we can draw no conclusions concerning mechanisms from the order of effectiveness of the various metal salts that we have tried.

What might be the physicochemical basis for the two forms of T7 DNA polymerase? A likely mechanism for such a conversion would be an irreversible modification of one of the two subunits. Several possibilities come to mind. An EDTA-inhibited proteolysis of either the gene 5 protein or thioredoxin would be compatible with the results we have presented. Form II exists as the precursor, and Form I is the product, of proteolytic cleavage. Proteolysis would not be readily reversible, and EDTA is known to inhibit some *E. coli* proteases (47). However, either the polymerase itself would have to serve as the protease or the contaminating protease must be present at a constant amount. The extent of proteolysis would have to be slight since no difference in mobility of either subunit was detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In support of the limited proteolysis hypothesis in the conversion is the finding that new polypeptides can be detected after dialysis of Form II of T7 DNA polymerase in the absence of EDTA. However, no causal relationship between the conversion and the apparent proteolysis has been demonstrated.

On the other hand, one need not invoke proteolysis in the initial step leading to the conversion of Form II to Form I. For example, the conversion need not be enzyme catalyzed, but rather the result of modification of a chemically reactive site in one of the two subunits of T7 DNA polymerase. In any case, the possibility of regulation arises in the consideration of any of these general conversion mechanisms.

One can only speculate on the physiological significance of the two forms of T7 DNA polymerase. It is our contention that high exonuclease activity and absence of polymerase activity at nicks are properties suited to the faithful replication of a duplex DNA. Therefore, Form II of T7 DNA polymerase is most likely the preferred polymerase for the synthesis of DNA at the replication fork. In fact our initial attempts to reconstitute lagging strand synthesis using Form I of T7 DNA polymerase were frustrated by the capacity of this enzyme to displace Okazaki fragments before they could be ligated, a complication that is described in an accompanying paper (41). Our initial concern that Form II, if indeed it is the form that functions at the replication fork, should have the capacity to be stimulated by gene 4 protein to copy duplex DNA proved to be without foundation. As will be shown in the third paper of this series (24), the lack of stimulation of Form II by gene 4 protein at nicks reflects only the inability of this polymerase to generate a single strand to which the gene 4 protein can bind. In fact, presented with such a preformed replication fork, Form II of T7 DNA polymerase responds to gene 4 protein to a greater extent than does Form I. Similarly, the inability of Form II to initiate synthesis at nicks is of no consequence for initiation at the primary origin. In this step of DNA replication, T7 RNA polymerase synthesizes a primer and simultaneously creates a single-stranded lagging strand that Form II of T7 DNA polymerase and gene 4 protein can use effectively (1).

Is Form I of T7 DNA polymerase, then, an artifact that arises during purification since Form II appears to be the predominant, if not exclusive, species present in lysates of T7-infected *E. coli* cells? Certain properties of Form I make it a more suitable candidate than Form II for some reactions of DNA metabolism. In the following paper (23), we suggest the involvement of Form I in steps that call for strand displacement synthesis such as the processing of concatemers of T7 DNA and in recombination.

Acknowledgments—We thank Steven Matson for the preparation of Form II of T7 DNA polymerase (greater than 95% pure) and Bess Swartz for her secretarial assistance.

REFERENCES

1. Tabor, S., Engler, M. J., Fuller, C. W., Lechner, R. L., Matson, S. W., Romano, L. J., Saito, H., Tamanoi, F., and Richardson, C. C. (1981) in *Structure and DNA-Protein Interactions of Replication Origins. ICN-UCLA Symposia on Molecular and Cellular Biology* (Ray, D. S., ed) Vol. XXII, pp. 387–408, Academic Press, New York
2. Grippo, P., and Richardson, C. C. (1971) *J. Biol. Chem.* **246**, 6867–6873
3. Oley, S. L., Stratling, W.. and Knippers, R. (1971) *Eur. J. Biochem.* **23**, 497–504
4. Modrich, P., and Richardson, C. C. (1975) *J. Biol. Chem.* **250**, 5515–5522
5. Scherzinger, E., and Seiffert, D. (1975) *Mol. Gen. Genet.* **141**, 213–232
6. Adler, S., and Modrich, P. (1979) *J. Biol. Chem.* **254**, 11605–11614

7. Modrich, P., and Richardson, C. C. (1975) *J. Biol. Chem.* **250**, 5508–5514
8. Mark, D. F., and Richardson, C. C. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 780–784
9. Mark, D. F., Chase, J. W., and Richardson, C. C. (1977) *Mol. Gen. Genet.* **155**, 145–152
10. Hori, K., Mark, D. F., and Richardson, C. C. (1979) *J. Biol. Chem* **254**, 11598–11604
11. Hori, K., Mark, D. F., and Richardson, C. C. (1979) *J. Biol. Chem.* **254**, 11591–11597
12. Kolodner, R., and Richardson, C. C. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 1525–1529
13. Scherzinger, E., Lanka, E., Morelli, G., Seiffert, D., and Yuki, A. (1977) *Eur. J. Biochem.* **72**, 543–558
14. Tabor, S., and Richardson, C. C. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 205–209
15. Scherzinger, E., Lanka, E., and Hillenbrand, G. (1977) *Nucleic Acids Res.* **4**, 4151–4163
16. Hillenbrand, G., Morelli, G., Lanka, E., and Scherzinger, E. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 449–459
17. Romano, L. J., and Richardson, C. C. (1979) *J. Biol. Chem.* **254**, 10476–10482
18. Romano, L. J., and Richardson, C. C. (1979) *J. Biol. Chem.* **254**, 10483–10489
19. Kolodner, R., and Richardson, C. C. (1978) *J. Biol. Chem.* **253**, 574–584
20. Kolodner, R., Masamune, Y., LeClerc, J. E., and Richardson, C. C. (1978) *J. Biol. Chem.* **253**, 566–573
21. Fischer, H., and Hinkle, D. C. (1980) *J. Biol. Chem.* **255**, 7956–7964
22. Stratling, W., and Knippers, R. (1973) *Nature (Lond.)* **245**, 195–197
23. Lechner, R. L., Engler, M. J., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11174–11184
24. Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11185–11196
25. Moses, R. E., and Richardson, C. C. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **67**, 674–681
26. Studier, F. W. (1969) *Virology* **39**, 562–574
27. Studier, F. W. (1973) *J. Mol. Biol.* **79**, 227–236
28. Simon, M. N., and Studier, F. W. (1973) *J. Mol. Biol.* **79**, 249–265
29. Richardson, C. C. (1966) *J. Mol. Biol.* **15**, 49–61
30. Hutchison, C. A., III, and Sinsheimer, R. L. (1966) *J. Mol. Biol.* **18**, 429–447
31. Campbell, J. L., Richardson, C. C., and Studier, F. W. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2276–2280
32. Weiss, B., Live, T. R., and Richardson, C. C. (1968) *J. Biol. Chem.* **243**, 4530–4542
33. Richardson, C. C. (1971) *Nucleic Acids Res.* **2**, 815–828
34. Hinkle, D. C., and Richardson, C. C. (1974) *J. Biol. Chem.* **249**, 2974–2984
35. Hershfield, M. S., and Nossal, N. G. (1972) *J. Biol. Chem.* **247**, 3393–3404
36. Hinkle, D. C., and Richardson, C. C. (1975) *J. Biol. Chem.* **250**, 5523–5529
37. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
38. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680–685
39. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
40. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
41. Engler, M. J., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11197–11205
42. Muzyczka, N., Poland, R. L., and Bessman, M. J. (1972) *J. Biol. Chem.* **247**, 7116–7122
43. Bessman, M. J., Muzyczka, N., Goodman, M. F., and Schnaar, R. L. (1974) *J. Mol. Biol.* **88**, 409–421
44. Brutlag, D., and Kornberg, A. (1972) *J. Biol. Chem.* **247**, 241–248
45. Lo, K-Y., and Bessman, M. J. (1976) *J. Biol. Chem.* **251**, 2480–2486
46. Gillin, F. D., and Nossal, N. G. (1976) *J. Biol. Chem.* **251**, 5219–5224
47. Goldberg, A. L., Swamy, K. H. S., Chung, C. H., and Larimore, F. S. (1981) *Methods Enzymol.* **80**, 680–702
48. Saito, H., and Richardson, C. C. (1981) *Cell* **27**, 533–542